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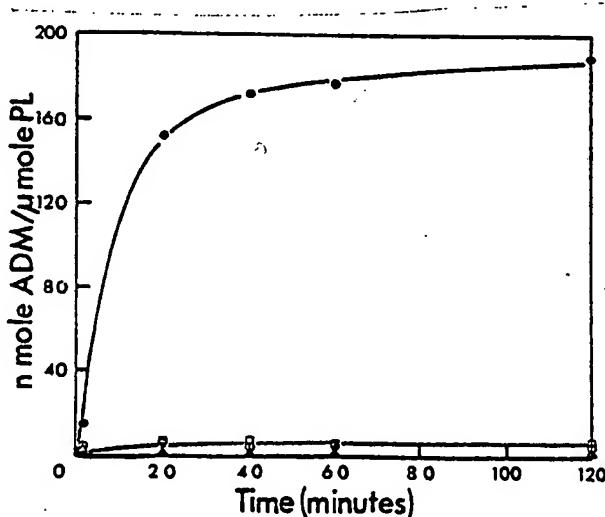
(57) Abstract

Methods for encapsulating ionizable antineoplastic agents in liposomes using transmembrane potentials. Trapping efficiencies approaching 100% and rapid loading are readily achieved. Dehydration protocols which allow liposomes to be conveniently used in the administration of antineoplastic agents in a clinical setting are also provided. In accordance with other aspects of the invention, transmembrane potentials are used to reduce the rate of release of ionizable drugs from lip-

ENCAPSULATION OF ANTINEOPLASTIC AGENTS IN LIPOSOMES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to antineoplastic agents and in particular to the encapsulation of such agents in liposomes.

2. Description of the Prior Art

As has been established by various investigators, cancer therapy employing antineoplastic agents can in many cases be significantly improved by encapsulating the antineoplastic agent in liposomes, rather- than administrating the agent directly into the body. See, for example, Forssen, et. al., (1983), Cancer Res., 43:546; Gabizon, et. al., (1982), Cancer Res., 42:4734; and Olson, et. al., (1982), Eur. J. Cancer Clin. Oncol., 18:167; but see Abra, et. al., (1983), Cancer Chemother. Pharmacol., 11:98. Incorporation of such agents in liposomes changes their antitumor activities, clearance rates, tissue distributions, and toxicities compared to direct administration. See, for example, Rahman, et. al., (1982), Cancer Res., 42:1817; Rosa, et. al., (1982) in Transport in Biomembranes: Model Systems and Reconstitution, R. Antolini et. al., ed., Raven Press, New York. 243-256; Rosa, et. al., (1983), Pharmacology, 26:221; Forssen, et. al., supra; Gabizon, et. al., supra; and Olson, et. al., supra. For example, it is well known that the cardiotoxicity of the anthracycline antibiotics daunorubicin and doxorubicin (adriamycin) and their pharmaceutically acceptable derivatives and salts can be significantly reduced through liposome encapsulation. See, for example, Forssen, et. al., supra; Olson, et. al., supra; and Rahman, et. al., supra. Also, incorporation of highly toxic antineoplastic agents in liposomes can reduce the risk of exposure to such agents by persons involved in their administration.

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The use of liposomes to administer antineoplastic agents has raised problems with regard to both drug encapsulation and drug release during therapy. With regard to encapsulation, there has been a continuing need to increase trapping efficiencies so as to
5   minimize the lipid load presented to the patient during therapy. In addition, high trapping efficiencies mean that only a small amount of drug is lost during the encapsulation process, an important advantage when dealing with the expensive drugs currently being used in cancer therapy.
10   As to drug release, many antineoplastic agents, such as adriamycin, have been found to be rapidly released from liposomes after encapsulation. Such rapid release diminishes the beneficial effects of liposome encapsulation and thus, in general, is undesirable. Accordingly, there have been continuing efforts by
15   workers in the art to find ways to reduce the rate of release of antineoplastic agents and other drugs from liposomes.

In addition to these problems with encapsulation and release, there is the overriding problem of finding a commercially acceptable way of providing liposomes containing antineoplastic
20   agents to the clinician. Although the production and loading of liposomes on an "as needed" basis is an acceptable procedure in an experimental setting, it is, in general, unsatisfactory in a clinical setting. Accordingly, there is a significant and continuing need for methods whereby liposomes, with or without
25   encapsulated drugs, can be shipped, stored and in general moved through conventional commercial distribution channels without substantial damage.

SUMMARY OF THE INVENTION

In view of the above state of the art, it is an object of the
30   present invention to provide improved methods for encapsulating antineoplastic agents in liposomes. It is an additional object of the invention to provide methods for reducing the rate of release of antineoplastic agents and other biologically-active agents from liposomes. It is a further object of the invention to provide
35   methods for dehydrating liposomes, either before or after the loading of antineoplastic agents, so that the liposomes can be

stored, shipped, and commercially distributed without substantial damage.

To achieve these and other objects, the invention, in accordance with one of its aspects, provides a method for loading
5   liposomes with ionizable antineoplastic agents wherein a transmembrane potential is created across the walls of the liposomes and the antineoplastic agent is loaded into the liposomes by means of the transmembrane potential.

The method can be used with essentially any antineoplastic
10   agent which can exist in a charged state when dissolved in an aqueous medium (e.g., organic compounds which include an amino group which can be protonated). Preferably, the agent should be relatively lipophilic so that it will partition into the liposome membranes. Multiple antineoplastic agents can be loaded either
15   simultaneously or sequentially into liposomes using the method. Also, the liposomes into which the ionizable antineoplastic agents are loaded can themselves be pre-loaded with other antineoplastic agents or other drugs using conventional encapsulation techniques (e.g., by incorporating the drug in the buffer from which the
20   liposomes are made).

In accordance with other aspects of the invention, a method is provided for reducing the rate of release of an ionizable antineoplastic agent or other ionizable biologically-active agent from liposomes wherein a transmembrane potential, oriented to
25   retain the agent in the liposomes, is generated across the liposome membranes. As described in detail below, it has been surprisingly found that such a transmembrane potential is capable of producing a ten-fold reduction in the rate of release of ionizable drugs, such as adriamycin, from liposomes. The method
30   can be used with essentially any ionizable material which can be encapsulated in a liposome. The transmembrane potential can be generated after encapsulation or can be the same transmembrane potential used to load the liposomes in accordance with the encapsulation technique described above.

35   In accordance with further of its aspects, the invention provides various dehydration protocols which allow liposomes to be

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conveniently used in the administration of antineoplastic agents in a clinical setting. In accordance with certain of those protocols, antineoplastic agents are loaded into liposomes and the resulting preparation is dehydrated so that it can be conveniently 5 stored, shipped or otherwise handled without substantial leakage of the antineoplastic agent from the liposomes.

In accordance with certain embodiments of this protocol, the dehydration is done in the presence of one or more protective sugars. Preferably, the one or more sugars are present at both 10 the inside and outside surfaces of the liposome membranes. Further, it is preferred that the sugars be selected from the group consisting of trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose. The dehydration is 15 done under vacuum and can take place either with or without prior freezing of the liposome preparation.

In accordance with other embodiments of this protocol, the dehydration is done without the use of a protective sugar. In this case, the dehydration must be done without prior freezing, 20 the liposomes used to encapsulate the antineoplastic agent must be of the type which have multiple lipid layers, and the liposome preparation cannot be completely dehydrated, but rather between about 2% and about 5% of the original water in the preparation must be left in the preparation at the end of the dehydration 25 process. In terms of moles of water per mole of lipid, this means that between about 12 and about 35 moles water/mole lipid should be present in the dehydrated preparation.

In accordance with other dehydration protocols of the present invention, antineoplastic agents are loaded into liposomes which 30 have previously been dehydrated for shipping, storage and the like, and then rehydrated. The dehydration is performed following the procedures discussed above, and the loading of the antineoplastic agents is performed using the transmembrane potential method described above. The transmembrane potential can 35 be generated either before or after the dehydration step, as desired.

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In addition to the foregoing methods, the invention also provides the products produced by practicing the methods. That is, it provides: 1) pharmaceutical preparations comprising an antineoplastic agent which has been loaded into liposomes by means

5   of a transmembrane potential; 2) stabilized pharmaceutical preparations comprising an ionizable biologically-active agent encapsulated in liposomes having a transmembrane potential across their membranes so as to reduce the rate of release of the agent from the liposomes; 3) dehydrated pharmaceutical preparations

10 comprising antineoplastic agents encapsulated in liposomes; and 4) pharmaceutical preparations comprising antineoplastic agents which have been loaded into previously dehydrated liposomes by means of a transmembrane potential.

The attainment of the foregoing and other objects and

15 advantages of the present invention is described fully below in connection with the description of the preferred embodiments of the invention.

As used herein, the terms "pharmaceutical preparation," "ionizable antineoplastic agent," and "ionizable

20 biologically-active agent" have the following meanings: pharmaceutical preparation means a composition of matter suitable for administration to humans or animals and comprising a biologically active material and appropriate buffers, diluents, carriers, and the like; ionizable antineoplastic agent means an

25 antineoplastic agent which can exist in a charged state when dissolved in an aqueous medium; and ionizable biologically-active agent means a biologically-active agent which can exist in a charged state when dissolved in an aqueous medium.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1 shows the uptake of adriamycin (ADM) into large unilamellar vesicles (LUVs) in the presence and absence of a Na^+/K^+ diffusion potential. Egg-PC LUVs (1 umol phospholipid/ml) were incubated at 20°C in the presence of 0.2 mM adriamycin. Drug uptake was quantitated as described below in Materials and

35 Methods. Experimental conditions were as follows: open triangles — vesicles with 169 mM potassium glutamate, 20 mM Hepes (pH 7.5)

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in the internal and external medium; open circles -- vesicles with 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) in the internal and external medium plus valinomycin; open squares -- vesicles having 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) in the
5   internal medium and 150 mM NaCl, 20 mM Hepes (pH 7.5) in the external medium; solid circles -- vesicles having 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) in the internal medium and 150 mM NaCl, 20 mM Hepes (pH 7.5) in the external medium plus valinomycin.

10   Figure 2 shows the uptake of vinblastine (VINB) into large unilamellar vesicles in the presence and absence of a Na^+/K^+ diffusion potential. Egg-PC LUVs (1 umol phospholipid/ml) were incubated at 20°C in the presence of 0.2 mM vinblastine under the following conditions: open triangles -- 169 mM potassium
15   glutamate, 20 mM Hepes (pH 7.5) as the interior and exterior buffers; open circles -- 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) as the interior and exterior buffers plus valinomycin; open squares -- 150 mM NaCl, 20 mM Hepes (pH 7.5) in the exterior medium and 169 mM potassium glutamate in the vesicle interior;
20   solid circles -- 150 mM NaCl, 20 mM Hepes (pH 7.5) in the exterior medium and 169 mM potassium glutamate in the vesicle interior plus valinomycin.

Figure 3 shows the uptake of adriamycin (ADM) into egg-PC LUVs having Na^+/K^+ gradients across their membranes as a function
25   of the initial free adriamycin concentration (solid circles). The open circles show the percentage of the total available drug which was accumulated. The vesicles (1 mM phospholipid) were incubated at 20°C with the indicated concentrations of adriamycin for 2 hours in the presence of valinomycin.

30   Figure 4 shows the uptake of adriamycin (ADM) into egg-PC-cholesterol LUV systems in the presence of Na^+/K^+ transmembrane chemical gradients and valinomycin at (A) 20°C and (B) 37°C. The vesicles (1 umol total lipid/ml) were incubated at 20°C or 37°C for 2 hours in the presence of 0.2 mM adriamycin.
35   The molar ratios of egg-PC to cholesterol employed were: solid

circles -- 1:0; open circles -- 9:1; solid squares -- 3:1; and open squares -- 1:1.

Figure 5 shows the uptake of adriamycin (ADM) into DPPC-cholesterol (1:1) LUVs (1 mM lipid) in the presence of Na^+/K^+ transmembrane chemical gradients after incubations in the presence of 0.2 mM adriamycin at various temperatures: open circles -- 20°C, in the presence of valinomycin; solid squares -- 37°C, in the presence of valinomycin; solid circles -- 37°C, in the absence of valinomycin; open squares -- 60°C, in the presence of valinomycin; solid downward facing triangles -- 60°C, in the absence of valinomycin; solid upward facing triangles -- Egg-PC LUVs (1 mM) at 37°C, in the absence of valinomycin; open downward facing triangles -- Egg-PC LUVs at 60°C, in the absence of valinomycin.

Figure 6 shows the uptake of adriamycin (ADM) into egg-PC/egg-PS LUVs in the presence of Na^+/K^+ chemical gradients after incubation at 20°C in the presence of 0.2 mM adriamycin: open triangles -- egg-PC/egg-PS (4:1), in the presence of valinomycin; solid triangles -- egg-PC/egg-PS (4:1), in the absence of valinomycin; open squares -- egg-PC/egg-PS (9:1), in the absence of valinomycin; solid squares -- egg-PC/egg-PS (20:1), in the absence of valinomycin; open circles -- egg-PC/egg-PS (50:1), in the absence of valinomycin; solid circles -- uptake into the egg-PC/egg-PS (4:1) system in the absence of an Na^+/K^+ gradient (K^+ buffer inside and out).

Figure 7 shows the uptake of adriamycin into LUVs exhibiting a transmembrane pH gradient at 20°C (A) and 37°C (B). Experimental conditions were: 2 mM lipid, 0.2 mM adriamycin in the absence (solid symbols) and presence (open symbols) of the proton ionophore CCCP (10 uM). The interior buffer composition was 150 mM KOH, 175 mM glutamic acid (pH 4.6), whereas the exterior buffer composition consisted of 150 mM KOH, 125 mM glutamic acid, 30 mM NaCl (pH 7.5). Lipid compositions were EPC (circles) and EPC/cholesterol at a molar ratio of 1:1 (squares).

Figure 8 shows the release of adriamycin from EPC (solid circle, open circle) and EPC/cholesterol at a molar ratio of 1:1

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(solid square, open square) LUVs at 37°C. Adriamycin was sequestered into vesicles in response to a pH gradient at lipid and drug concentrations of 5.0 and 0.5 mM, respectively. Free adriamycin was separated from vesicle-associated drug by gel
5   filtration chromatography employing columns equilibrated in buffers adjusted to pH 4.6 (panel A) or pH 7.5 (panel B) which contained 150 mM K^+ (closed symbols) or 180 mM Na^+ (open symbols).

Figure 9 shows the transmembrane potentials generated by a pH gradient for control vesicles (squares) and dehydrated/rehydrated
10   vesicles (circles). Vesicles with a pre-existing proton gradient were maintained at 4°C for 24 hours (control) or dehydrated in the presence of 250 mM trehalose under high vacuum for the same period of time. The potential observed in the vesicles upon rehydration was determined in the absence of CCCP (open circles and squares),
15   or with 20 uM CCCP present (solid circles and squares), using the probe ^3H-tetraphenylphosphonium bromide. The transmembrane potentials observed in vesicles without a pH gradient in the presence and absence of CCCP is shown by the solid and open triangles, respectively.

20   Figure 10 shows the transmembrane potentials generated by a Na^+/K^+ chemical gradient for control vesicles (squares) and dehydrated/rehydrated vesicles (circles). Vesicles with a pre-existing Na^+/K^+ gradient were maintained at 4°C for 24 hours (control) or dehydrated in the presence of 250 mM trehalose under
25   high vacuum for the same period of time. The potential observed in the vesicles upon rehydration was determined in the absence of valinomycin (solid circles and squares), or with 0.5 ug/umole phospholipid valinomycin present (open circles and squares), using the probe ^3H-tetraphenylphosphonium bromide. The transmembrane
30   potentials observed in vesicles having potassium glutamate on both sides of the membrane in the presence and· absence of valinomycin is shown by the open and solid triangles, respectively.

Figure 11 illustrates the use of a transmembrane potential to load adriamycin into previously dried vesicles. Vesicles with a
35   pre-existing Na^+/K^+ gradient were dehydrated for 24 hours in the presence of 250 mM trehalose. Following rehydration the ability

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of the vesicles to accumulate adriamycin in the presence (open circles), or absence (solid circles) of valinomycin (0.5 ug/umole phospholipid) was measured. Control vesicles maintained at 4°C for the same period were also tested in the presence (open squares) or absence (solid squares) of valinomycin.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above, the present invention relates to the encapsulation of antineoplastic agents in liposomes.

The liposomes in which the antineoplastic agents are encapsulated can have a variety of compositions and internal contents, and can be in the form of multilamellar, unilamellar, or other types of liposomes or, more generally, lipid-containing particles, now known or later developed. For example, the lipid-containing particles can be in the form of steroidal liposomes, stable plurilamellar liposomes (SPLVs), monophasic vesicles (MPVs), or lipid matrix carriers (LMCs) of the types disclosed in copending and commonly assigned U.S. Patent Applications Serial Nos. 476,496, 521,176, 591,576 and 599,691, filed March 24, 1983, August 8, 1983, March 20, 1984, and April 12, 1984, respectively, the pertinent portions of which are incorporated herein by reference.

The liposomes can be prepared by any of the techniques now known or subsequently developed for preparing liposomes. For example, the liposomes can be formed by the conventional technique for preparing multilamellar liposomes (MLVs), that is, by depositing one or more selected lipids on the inside walls of a suitable vessel by dissolving the lipids in chloroform and then evaporating the chloroform, adding the aqueous solution which is to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and swirling or vortexing the resulting lipid suspension to produce the desired liposomes.

Alternatively, techniques used for producing large unilamellar liposomes (LUVs), such as, reverse-phase evaporation, infusion procedures, and detergent dilution, can be used to produce the liposomes. A review of these and other methods for producing liposomes can be found in the text *Liposomes*, Marc J.

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Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1, the pertinent portions of which are incorporated herein by reference. See also Szoka, Jr., et al., (1980) Ann. Rev. Biophys. Bioengr., 9:467, the pertinent portions of which are also incorporated herein by reference. A particularly preferred method for preparing LUVs is described in commonly assigned and copending U.S. Patent Application Serial No. 622,690, filed June 20, 1984, and entitled "Extrusion Technique for Producing Unilamellar Vesicles," the pertinent portions of which are incorporated herein by reference.

As other alternatives, the liposomes can be produced in accordance with the procedures described in U.S. Patent Applications Serial Nos. 476,496, 521,176 and 599,691, referred to above. Also, rather than using liposomes per se, other lipid-containing particles, such as those described in U.S. Patent Application Serial No. 591,576, referred to above, can be used in the practice of the present invention. Furthermore, if desired, the liposomes or lipid-containing particles which are used to carry the antineoplastic agents can be given a more uniform size distribution by subjecting them to the process of commonly assigned and copending U.S. Patent Application Serial No. 622,502, filed June 20, 1984, and entitled "Liposomes Having Defined Size Distributions," the pertinent portions of which are incorporated herein by reference.

As discussed above, in accordance with one of its aspects, the present invention provides a method for loading liposomes with ionizable antineoplastic agents wherein a transmembrane potential is created across the walls of the liposomes and the antineoplastic agent is loaded into the liposomes by means of the transmembrane potential. The transmembrane potential is generated by creating a concentration gradient for one or more charged species (e.g., Na^+, K^+ and/or H^+) across the liposome membranes. The concentration gradient is created by producing liposomes having different internal and external media, i.e., internal and external media having different concentrations of the one or more charged species.

Specifically, liposomes are prepared which encapsulate a first medium having a first concentration of the one or more charged species. For a typical liposome preparation technique (see discussion above), this first medium will surround the

5   liposomes as they are formed, and thus the liposomes' original external medium will have the same composition as the first medium. To create the concentration gradient, the original external medium is replaced by a new external medium having a different concentration of the one or more charged species. The replacement

10   of the external medium can be accomplished by various techniques, such as, by passing the liposome preparation through a gel filtration column, e.g., a Sephadex column, which has been equilibrated with the new medium, or by centrifugation, dialysis, or related techniques.

15   Depending upon the permeability of the liposome membranes, the full transmembrane potential corresponding to the concentration gradient will either form spontaneously or a permeability enhancing agent, e.g., an ionophore, such as, valinomycin, may have to be added to the bathing medium. (Note

20   that, if desired, the permeability enhancing agent can be removed from the preparation after loading has been completed using chromatography or other techniques.) In either case, a transmembrane potential having a magnitude defined by the Nernst equation will appear across the liposomes' membranes.

25   In accordance with the invention, it has been found that this transmembrane potential can be used to load ionizable antineoplastic agents into the liposomes. Specifically, once liposomes having a concentration gradient and thus a transmembrane potential of the appropriate orientation have been prepared, the

30   process of loading antineoplastic agents into the liposomes reduces to the very simple step of adding the agent to the external medium. Once added, the transmembrane potential will automatically load the agent into the liposomes. Moreover, as described in detail in Example 1 below, the loading is not only

35   simple, but is also extremely efficient. As described in that example, it has been found that trapping efficiencies for

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antineoplastic agents of 95% and higher can be readily achieved
with the transmembrane potential loading technique.

The transmembrane potential loading method can be used with
essentially any antineoplastic agent which can exist in a charged
5   state when dissolved in an appropriate aqueous medium (e.g.,
organic compounds which include an amino group which can be
protonated).   Preferably,   the   agent   should   be   relatively
lipophilic so that it will partition into the liposome membranes.
Examples of some of the antineoplastic agents which can be loaded
10   into liposomes by this method include doxorubicin, mitomycin,
bleomycin, daunorubicin, streptozocin, vinblastine, vincristine,
mechlorethamine   hydrochloride,   melphalan,   cyclophosphamide,
triethylenethiophosphoramide, carmustine, lomustine, semustine,
fluorouracil, hydroxyurea, thioguanine, cytarabine, floxuridine,
15   decarbazine, cisplatin and procarbazine.

In addition to loading a single antineoplastic agent, the
method can be used to load multiple antineoplastic agents, either
simultaneously or sequentially. Also, the liposomes into which
the ionizable antineoplastic agents are loaded can themselves be
20   pre-loaded with other antineoplastic agents or other drugs using
conventional encapsulation techniques (e.g., by incorporating the
drug in the buffer from which the liposomes are made). Since the
conventionally loaded materials need not be ionizable, this
approach   provides   great   flexibility   in   preparing
25   liposome-encapsulated "drug cocktails" for use in cancer
therapies. Indeed, essentially all types of anti-cancer drugs can
be pre-loaded, at least to some extent, in either the lipid or
aqueous portion of the liposomes. Of course, if desired, one or
more of the ionizable drugs listed above can be pre-loaded and
30   then the same or a different drug added to the liposomes using the
transmembrane potential approach.

Turning now to the aspects of the invention relating to
reducing the rate of release of an ionizable antineoplastic agent
or other ionizable biologically-active agent drug from liposomes,
35   it has been surprisingly found that the rate of release can be
markedly reduced by creating a transmembrane potential across the

liposome membranes which is oriented to retain the agent in the
liposomes. That is, for an agent which is positively charged when
ionized, a transmembrane potential is created across the liposome
membranes which has an inside potential which is negative relative
5   to the outside potential, while for a agent which is negatively
charged, the opposite orientation is used.

As with the transmembrane loading aspects of the invention,
the transmembrane potentials used to reduce the rate of drug
release are created by adjusting the concentrations on the inside
10   and outside of the liposomes of a charged species such as Na^+, K^+
and/or H^+. Indeed, if the liposomes have been loaded by means of
a transmembrane potential produced by such a concentration
gradient, simply keeping the liposomes in an external medium which
will maintain the original concentration gradient will produce the
15   desired reduction in the rate of release. Alternatively, if a
transmembrane potential has not already been created across the
liposome membranes, e.g., if the liposomes have been loaded using
a conventional technique, the desired transmembrane potential can
be readily created by changing the composition of the external
20   medium using the exchange techniques described above.

The reduced rate of release aspect of the invention can be
used with essentially any ionizable biologically-active agent
which can be encapsulated in a liposome. In particular, the
technique can be used with the ionizable antineoplastic agents
25   listed above and with a variety of other ionizable drugs,
including such drugs as local anesthetics, e.g., dibucaine and
chlorpronazine; beta-adrenergic blockers, e.g., propanolol,
timolol and labetolol; antihypertensive agents, e.g., clonidine,
and hydralazine; anti-depressants, e.g., imipramine, amipriptyline
30   and doxepim; anti-convulsants, e.g., phenytoin; anti-emetics,
e.g., procainamide and prochlorperazine; antihistamines, e.g.,
diphenhydramine,   chlorpheniramine   and   promethazine;
anti-arrhythmic   agents,   e.g.,   quinidine   and   disopyramide;
anti-malarial   agents,   e.g.,   chloroquine;   and   analgesics   e.g.,
35   cocaine. In general, the largest reductions in the rate of
release will be seen for lipophilic materials since their normal

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rates of release are typically higher than those of non-lipophilic materials.

Turning next to the aspects of the invention relating to the dehydration protocols, two basic approaches are provided: 1) the liposomes can be loaded with antineoplastic agents (e.g., using conventional techniques or the transmembrane potential loading technique described above), dehydrated for purposes of storage, shipping, and the like, and then rehydrated at the time of use; or 2) pre-formed liposomes can be dehydrated for storage, etc., and then at or near the time of use, they can be rehydrated and loaded with an ionizable antineoplastic agent using the transmembrane potential loading technique described above.

In either case, the liposomes are preferably dehydrated using standard freeze-drying equipment or equivalent apparatus, that is, they are preferably dehydrated under reduced pressure. If desired, the liposomes and their surrounding medium can be frozen in liquid nitrogen before being dehydrated. Alternatively, the liposomes can be dehydrated without prior freezing, by simply being placed under reduced pressure. Dehydration without prior freezing takes longer than dehydration with prior freezing, but the overall process is gentler without the freezing step, and thus there is in general less damage to the liposomes and a corresponding smaller loss of the internal contents of the liposomes. Dehydration without prior freezing at room temperature and at a reduced pressure provided by a vacuum pump capable of producing a pressure on the order of 1 mm of mercury typically takes between approximately 24 and 36 hours, while dehydration with prior freezing under the same conditions generally takes between approximately 12 and 24 hours.

So that the liposomes will survive the dehydration process without losing a substantial portion of their internal contents, it is important that one or more protective sugars be available to interact with the liposome membranes and keep them intact as the water in the system is removed. A variety of sugars can be used, including such sugars as trehalose, maltose, sucrose, glucose, lactose, and dextran. In general, disaccharide sugars have been

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found to work better than monosaccharide sugars, with the disaccharide sugars trehalose and sucrose being most effective. Other more complicated sugars can also be used. For example, aminoglycosides, including streptomycin and dihydrostreptomycin, have been found to protect liposomes during dehydration.

The one or more sugars are included as part of either the internal or external media of the liposomes. Most preferably, the sugars are included in both the internal and external media so that they can interact with both the inside and outside surfaces of the liposomes' membranes. Inclusion in the internal medium is accomplished by adding the sugar or sugars to the buffer which becomes encapsulated in the liposomes during the liposome formation process. Since in most cases this buffer also forms the bathing medium for the finished liposomes, inclusion of the sugars in the buffer also makes them part of the external medium. Of course, if an external medium other than the original buffer is used, e.g., to create a transmembrane potential (see above), the new external medium should also include one or more of the protective sugars.

The amount of sugar to be used depends on the type of sugar used and the characteristics of the liposomes to be protected. As described in commonly assigned and copending U.S. Patent Application Serial No. 638,809, filed August 8, 1984, and entitled "Dehydrated Liposomes," the pertinent portions of which are incorporated herein by reference, persons skilled in the art can readily test various sugar types and concentrations to determine which combination works best for a particular liposome preparation. In general, sugar concentrations on the order of 100 mM and above have been found necessary to achieve the highest levels of protection. In terms of moles of membrane phospholipid, millimolar levels on the order of 100 mM correspond to approximately 5 moles of sugar per mole of phospholipid.

In the case of dehydration without prior freezing, if the liposomes being dehydrated are of the type which have multiple lipid layers and if the dehydration is carried to an end point where between about 2% and about 5% of the original water in the

preparation is left in the preparation, the use of one or more
protective sugars may be omitted.

Once the liposomes have been dehydrated, they can be stored
for extended periods of time until they are to be used. The
5   appropriate temperature for storage will depend on the make up of
the liposomes and the temperature sensitivity of whatever
materials have been encapsulated in the liposomes. For example,
as is known in the art, various antineoplastic agents are heat
labile, and thus dehydrated liposomes containing such agents
10   should be stored under refrigerated conditions so that the potency
of the agent is not lost. Also, for such agents, the dehydration
process is preferably carried out at reduced temperatures, rather
than at room temperature.

When the dehydrated liposomes are to be used, rehydration is
15   accomplished by simply adding an aqueous solution, e.g., distilled
water or an appropriate buffer, to the liposomes and allowing them
to rehydrate. The liposomes can be resuspended into the aqueous
solution by gentle swirling of the solution. The rehydration can
be performed at room temperature or at other temperatures
20   appropriate to the composition of the liposomes and their internal
contents.

If the antineoplastic agent which is to be administered was
incorporated into the liposomes prior to dehydration, and no
further composition changes are desired, the rehydrated liposomes
25   can be used directly in the cancer therapy following known
procedures for administering liposome encapsulated drugs.

Alternatively, using the transmembrane potential procedures
described above, ionizable antineoplastic agents can be
incorporated into the rehydrated liposomes just prior to
30   administration. In connection with this approach, the
concentration gradient used to generate the transmembrane
potential can be created either before dehydration or after
rehydration using the external medium exchange techniques
described above.

35   For example, liposomes having the same internal and external
media, i.e., no transmembrane potentials, can be prepared,

dehydrated, stored, rehydrated, and then the external medium can
be replaced with a new medium having a composition which will
generate transmembrane potentials, and the transmembrane
potentials used to load ionizable antineoplastic agents into the

5 liposomes. Alternatively, liposomes having internal and external
media which will produce transmembrane potentials can be prepared,
dehydrated, stored, rehydrated, and then loaded using the
transmembrane potentials.

Without intending to limit it in any manner, the present

10 invention will be more fully described by the following examples.
The materials and methods which are common to the various examples
are as follows.

Materials and Methods

Materials

15 Egg phosphatidylcholine (egg-PC or EPC) was isolated employ-
ing standard procedures (see, for example, Singleton, et al.,
(1965) Journal of the American Oil Chemical Society, 42:53) and
was more than 99% pure as determined by TLC. Egg PS was prepared
from egg-PC as described in Hope, et al., (1985), Biochim.

20 Biophys. Acta 812:55.

Cholesterol,   valinomycin,   vinblastine,   Hepes,
dipalmitoylphosphatidylcholine (DPPC), trehalose, CCCP, and salts
were obtained from the Sigma Chemical Company (St. Louis, Mo.).

Adriamycin was obtained either from Adria Laboratory

25 (Mississauga, Ontario) or from the Cancer Research Centre
(Vancouver, B.C.). Methotrexate and cytosine arabinoside were
also obtained from the Cancer Research Centre.

Tritiated methotrexate, DPPC and methyltriphenylphosphonium,
as well as $^{22}Na^+$, ^3H-inulin, ^{14}C-inulin, ^3H-tetraphenylphosphonium

30 bromide and ^3H-H_2O were obtained from New England Nuclear
(Lachine, Quebec). Tritiated cytosine arabinoside was obtained
from Amersham (Oakville, Ontario).

Reagents

Potassium glutamate, KCl and NaCl buffers were prepared in a

35 20 mM Hepes buffer adjusted to pH 7.5 with NaOH. The solutions
were adjusted to a common osmolarity of 310 mOsm/kg, which

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corresponded to KCl, NaCl and potassium glutamate concentrations
of 150, 150 and 169 mM, respectively.

Vesicle Preparation

Vesicles were prepared using the extrusion techniques
described in U.S. Patent Applications Serial Nos. 622,690 and
622,502, referred to above. A complete description of the
techniques used appears in those applications and is incorporated
herein by reference. The technique is also described in Hope, et
al., (1985) Biochim. Biophys. Acta, 812:55-65. Vesicles prepared
by these techniques will be referred to herein as either ETVs,
i.e, Extrusion Technique Vesicles or LUVs, i.e., Large Unilamellar
Liposomes.

Briefly, dry lipid films were hydrated with the appropriate
buffers to produce large multilamellar vesicles at concentrations
ranging between 25 and 200 umol phospholipid/ml. Where used,
$^{22}Na^+$ (5 uCi) or ^{3}H-inulin (5 uCi) was added to the dry lipid
prior to hydration.

The mixture was dispersed by vortexing and then passed ten
times through two stacked polycarbonate filters of 100 nm pore
size (Nuclepore, Inc., Pleasanton, CA) using a pressure of, for
example, 250 psi. For the experiments of Examples 1B and 2B, the
dispersions were frozen in liquid nitrogen and thawed 5 times
prior to extrusion through the polycarbonate filters. The
resulting vesicles had an average diameter of 103 nm and a trapped
volume of approximately 1.5 ul/umol phospholipid. For the
remaining experiments of Examples 1 and 2, the initial extrusion
was followed by two freeze-thaw cycles in liquid nitrogen, after
which the vesicles were passed through the filters five more
times. In this case, the vesicles had an average diameter of 90
nm and a trapped volume of again approximately 1.5 ul/umol
phospholipid.

Unencapsulated $^{22}Na^+$ or ^{3}H-inulin was removed by passing the
vesicles through a column (1.4 x 10 cm) of either Sephadex G-50
for removal of $^{22}Na^+$ or Ultragel AcA 34 for removal of ^{3}H-inulin.
This procedure generally diluted the phospholipid content of the
sample by approximately 50%.

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Dehydration.

Samples (1 ml) were dried in 10 ml Kimex tubes at room temperature under high vacuum using a Virtis Freeze Drier (Gardiner, N.Y.). In some cases, the samples were frozen in liquid nitrogen prior to dehydration. In either case, the reduced pressure dehydration process was carried out for approximately 24 hours.

Rehydration

Following dehydration and storage for periods ranging from 1 to 7 days, the samples were rehydrated with distilled water (900 ul) and the vesicles dispersed by gentle vortexing.

Assays

Phospholipids were quantified by determination of inorganic phosphorus as described by Chen, et al., (1956) Anal. Chem. 28:1756. See also Bottcher, et al., (1961) Anal. Chima. Acta, 24203. Alternatively, in some cases, liquid scintillation counting to quantitate [^{3}H]DPPC (0.05 uCi/umol lipid) was performed.

Adriamycin was quantitated by mixing an aliquot of the vesicle suspension with 0.5% Triton X-100 (which disrupted the vesicles and released the trapped drug) and monitoring the absorbance at 480 nm employing a Pye Unicam SP8-200 spectrophotometer. Vinblastine was assayed by determining the absorbance at 265 nm of the suspension dissolved in 94% ethanol.

The various tritiated compounds and ^{14}C-inulin were counted in a Phillips PW 4700 liquid scintillation counter, while ^{22}Na$^+$ was quantified by gamma counting on a Beckman Gamma 800.

Example 1

Loading of Ionizable Antineoplastic Agents

Into Liposomes Using Transmembrane Potentials

This example illustrates the active loading of ionizable antineoplastic agents into liposomes using transmembrane potentials.

Part A of the example illustrates the loading of the anti-cancer drugs adriamycin and vinblastine into liposomes using a Na$^+$/K$^+$ gradient to generate transmembrane potentials; Part B

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illustrates the loading of adriamycin using a pH gradient; and
Part C illustrates the use of transmembrane potentials to load
adriamycin into liposomes which previously have been passively
loaded with either methotrexate or cytosine arabinoside.

5   - Part A
           Active Loading Using Na^+/K^+ Gradients

           Membrane potentials were generated by forming egg-PC LUVs in
the potassium glutamate buffer (described above) and subsequently
exchanging the untrapped buffer for the NaCl buffer (described
10   above) employing Sephadex G-50 desalting columns. Where employed,
the potassium ionophore, valinomycin (1 mg/ml ethanol), was added
to achieve a concentration of 0.5 ug per umol lipid. Membrane
potentials were determined using the membrane potential probe
methyltriphenylphosphonium (see Bally, et al., (1985), Biochim.
15   Biophys. Acta, 812:66; see also Example 4, infra, where the probe
tetraphenylphosphonium rather than methyltriphenylphosphonium is
used).

           Adriamycin (0.2 mM final concentration) and vinblastine (0.2
mM final concentration) were added to LUV dispersions with and
20   without Na^+/K^+ ion gradients and with and without valinomycin. At
various times, the non-sequestered drug was removed by passing
aliquots of the solution over 1 ml Sephadex G-50 columns. Lipid
and drug concentrations were then assayed using the procedures
described above. The results are shown in Figures 1 and 2.

25   As shown in Figure 1, in the absence of both a Na^+/K^+
gradient and valinomycin, low LUV-associated adriamycin levels
(less than 6 nmol adriamycin/umol phospholipid) are observed over
the 2 hour incubation period. However, in the presence of
valinomycin and the Na^+/K^+ gradient, a remarkable increase in the
30   amount of vesicle-associated adriamycin is observed. This uptake
is more than 75% complete within 20 minutes and reaches an
equilibrium level of 190 nmol adriamycin/umol phospholipid. This
indicates that 95% of the drug initially contained in the solution
is taken up by the vesicles, reflecting a corresponding trapping
35   efficiency of 95%. In terms of concentrations, this uptake

corresponding to an internal adriamycin concentration of approximately 127 mM.

As shown in Figure 2, vinblastine can also be accumulated into LUV systems in response to a membrane potential. In the presence of valinomycin and a Na^+/K^+ gradient, 40 nmol vinblastine/umol phospholipid is accumulated within 2 hours, as compared to little or no uptake in the absence of a Na^+/K^+ gradient. In addition, appreciable uptake is obtained in the presence of a Na^+/K^+ gradient even in the absence of valinomycin.

In sum, the results of Figures 1 and 2 reveal a remarkable ability of LUV systems to sequester adriamycin and vinblastine in response to transmembrane potentials. Under the conditions employed, the uptake levels achieved correspond to final transmembrane drug concentration gradients of 2×10^2 and 2×10^4 for vinblastine and adriamycin, respectively. Furthermore, these gradients were found to be stable for 48 hours or longer at 20°C.

Although not wishing to be bound by any particular theory of operation, one of the mechanisms involved in the observed uptake of adriamycin, vinblastine, and other ionizable antineoplastic agents in response to a Na^+/K^+ gradient may involve the pH gradient which is automatically generated in response to the Na^+/K^+ gradient due to the permeability of liposome membranes to H^+ ions. In accordance with this mechanism, the ionizable antineoplastic agent passes through the membrane in an uncharged state, with its internal and external concentrations being a function of the internal and external H^+ ion concentrations, the internal concentration of the agent being high when the internal H^+ concentration is high, and vice versa.

The efficiency of the transmembrane potential loading process was further characterized by varying the amount of drug available. As shown in Figure 3, increasing the initial adriamycin concentration from 0 to 10 mM, while maintaining a fixed vesicle concentration (1 mM phospholipid), revealed that the transmembrane potential driven adriamycin uptake process saturated at approximately 400 nmol adriamycin/umol phospholipid. Between 0 and 0.2 mM adriamycin, the uptake was proportional to the initial

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free concentration and was nearly quantitative (95% or higher
trapping efficiencies). Above 0.2 mM adriamycin, the trapping
efficiency was reduced due to the saturation of the uptake
process. However, high trapping efficiencies at these higher drug
5   concentrations could be readily achieved by the simple expedient
of increasing the vesicle concentration. For example, it was
found that incubation of 10 mM adriamycin in the presence of LUVs
corresponding to a 50 mM phospholipid concentration yielded uptake
levels of 196 nmol adriamycin/umol phospholipid, corresponding to
10   a 98% trapping efficiency.

Similar studies employing vinblastine revealed that uptake
levels saturated at 40 nmol vinblastine/umol phospholipid.
Trapping efficiencies approaching 100% could be achieved on
incubation of 0.2 mM vinblastine with a concentration of LUVs
15   corresponding to 5 mM phospholipid.

Since liposomal drug delivery systems commonly contain
equimolar levels of cholesterol and more saturated phospholipid to
reduce leakage of entrapped material induced by serum components
(see, for example, Gabizon, et al., (1982), Cancer Res., 42:4734;
20   Mayhew, et al., (1979), Cancer Treat. Rep., 63:1923; and
Papahadjopoulos, et al., (1980), Liposomes and Immunology (Tom and
Six, eds.), Elsevier, New York), an investigation was performed to
determine the influence of cholesterol on active trapping of
adriamycin into egg/PC LUV systems. As shown in Figure 4A, at
25   20°C, a stepwise increase in cholesterol content to achieve
equimolar egg-PC/cholesterol levels resulted in a corresponding
decrease in the rate of adriamycin accumulation. Rapid uptake,
however, still could be achieved by simply incubating the
vesicle-drug system at higher temperatures. As shown in Figure
30   4B, equilibrium uptake levels are achieved within 30 minutes at
37°C. This effect was most pronounced for the egg-PC/cholesterol
(1:1) system, where increasing the temperature from 20°C to 37°C
resulted in an increase of vesicle-associated adriamycin from 42
to 153 nmol/umol lipid after a 30 minute incubation. These
35   results indicate that it is important to have a rather "fluid"

bilayer for efficient transport of drugs into the vesicle interior.

The effects of increased saturation of the phospholipid acyl chains on transmembrane potential loading of adriamycin were examined by monitoring uptake into DPPC-cholesterol (1:1) LUVs. As shown in Figure 5, no uptake of adriamycin could be observed over 4 hours at 20°C. However, incubation at 60°C resulted in sequestered adriamycin levels of 150 nmol drug/umol lipid within 2 hours. Further, significant uptake (to approximately 60 nmol/umol lipid) was observed for these systems incubated in the absence of valinomycin. Similarly, for egg-PC LUVs, adriamycin uptake levels of 60 and 100 nmol drug/umol lipid at 37°C and 60°C, respectively, were achieved in the absence of valinomycin (Figure 5).

In addition to variations in acyl chain composition and cholesterol content, charged lipid species, which influence in vivo distribution and uptake processes, have also been incorporated into liposomal delivery systems. See, for example, Fraley, et al., (1981), Biochemistry, 20:6978; Jonah, et al., (1975), Biochim. Biophys. Acta, 401:336; and Mauk, et al., (1979), Proc. Natl. Acad. Sci. USA, 76:765. In order to demonstrate that transmembrane potential loading can used with such systems, the effects of the acidic (negatively charged) phospholipid, egg-PS, on transmembrane potential loading of adriamycin into egg-PC LUVs was examined. As shown in Figure 6, systems containing 20 mol% egg-PS exhibited drug uptake behavior in the presence of valinomycin which was virtually identical to that observed in the absence of egg-PS (Figure 1). However, in contrast to the pure egg-PC systems, significant uptake was observed in egg-PS containing LUVs in the absence of valinomycin. Increasing the egg-PS content from 2 to 20 mol% increased such uptake (2 hour incubation) from 30 to 78 nmol adriamycin/umol phospholipid.

Part B

Active Loading Using pH Gradients

Transmembrane pH gradients were generated by forming LUVs in 150 mM KOH, 175 mM glutamic acid (pH 4.6) and subsequently exchanging the untrapped buffer for 150 mM KOH, 125 mM glutamic

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acid, 30 mM NaCl (pH 7.5) employing Sephadex G-50 desalting
columns (Hope, et. al., (1985), Biochim. Biophys. Acta, 812:55).
Where employed, the proton ionophore CCCP (10 mM in ethanol) was
added to achieve a concentration of 10 uM.

5   Adriamycin (0.2 mM final concentration) was added to the LUV
dispersions. At various times, the nonsequestered drug was
removed by passing aliquots of the solution over 1 ml Sephadex
G-50 columns. Lipid and drug concentration were then assayed.

        Figure 7 demonstrates that LUVs composed of EPC and
10   EPC/cholesterol (1:1) displaying a transmembrane pH gradient
actively accumulate adriamycin in the absence of the proton
ionophore CCCP. At 20°C, 50 percent of maximum uptake was
observed at 15 minutes and less than 5 minutes for the
EPC/cholesterol and the EPC LUVs, respectively (Figure 7A). These
15   results are consistent with the relative proton permeabilities of
the two membrane systems. The percent trapping efficiencies
observed for the EPC and EPC/cholesterol vesicles were 98 and 72
percent, respectively, comparable to values reported above for the
Na^+/K^+ gradients. The slightly lower amount of uptake obtained
20   for the cholesterol containing LUVs may be due to the fact that
these vesicles exhibit slightly lower trapped volume values than
do EPC vesicles.

        Inclusion of CCCP in the incubation mixture resulted in
transient high level accumulation of adriamycin followed by a
25   gradual release of the drug from the vesicle interior. Since the
LUV systems are apparently sufficiently permeable to protons to
accumulate adriamycin in the absence of CCCP, it is likely that
addition of the ionophore breaks down the pH gradient thus
eliminating the energetic force maintaining the very large
30   transmembrane drug concentration gradient.

        Increasing the incubation temperature to 37°C increased the
rate of uptake for EPC/cholesterol LUVs (Figure 7B) as compared to
systems equilibrated at 20°C (Figure 7B). A slow release of
adriamycin after 20 minutes was observed for EPC vesicles at
35   elevated temperatures in the absence of CCCP, while rapid release
occurred when the ionophore was present. Again, these results

correspond to the relative proton permeabilities for the various LUV systems.

Part C
Active/Passive Loading -- "Drug Cocktails"

The water-soluble anticancer drugs, methotrexate and cytosine arabinoside, which do not exhibit lipophilic or cationic characteristics, were passively encapsulated in LUVs during the initial vesicle formation process.

Briefly, egg-PC LUVs (187 umol phospholipid/ml) were generated in the potassium glutamate buffer described above to which had been added 20 mg/ml cytosine arabinoside (2 uCi/ml [^{3}H]cytosine arabinoside) or 10 mg/ml methotrexate (2 uCi/ml [^{3}H]methotrexate). The vesicles were subsequently passed over a gel filtration column pre-equilibrated with the NaCl buffer described above, which removed untrapped drug and also established a Na$^+$/K$^+$ chemical gradient. Analysis of LUVs prepared in this manner revealed that 33% of the available cytosine arabinoside or methotrexate was encapsulated (see Table I).

Following the passive trapping of the methotrexate and cytosine arabinoside, adriamycin was actively loaded into the vesicles employing the Na$^+$/K$^+$ gradient and valinomycin. In particular, as shown in Table I, incubation of an aliquot of the vesicles (1.0 mM phospholipid) in the presence of 0.2 mM adriamycin resulted in the uptake of 98% of the available adriamycin. Trapping efficiencies and uptake levels observed for adriamycin were almost identical to those observed in the absence of passively trapped drug (compare Table I and Figures 1 and 2).

Example 2
Reduction in the Rate of Release of Charged Drugs
From Liposomes Using Transmembrane Potentials

This example illustrates the ability of a transmembrane potential to reduce the rate at which ionizable drugs are released from liposomes. Parts A and B of the example illustrate, respectively, the use of Na$^+$/K$^+$ gradients and pH gradients to generate the requisite transmembrane potentials.

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Part A

Use of Na^+/K^+ Gradients to Reduce Drug Release Rates

The rate of release of adriamycin from LUVs subsequent to
active trapping following the procedures of Example 1A was assayed
5   as follows: vesicles (10 mM phospholipid) containing adriamycin
were first passed over a 15 ml gel filtration column equilibrated
with either the NaCl or KCl buffers, described above, to remove
free drug. The eluate was then placed in a flow dialysis
apparatus equilibrated at 37°C. Flow rates were adjusted to
10   achieve total exchange of the sample compartment volume (50 ml) in
20 minutes or less. Aliquots (100 ul) were removed at various
times and untrapped material was separated employing 1 ml gel
filtration columns. The sample was then assayed for adriamycin
and phospholipid.

15   The results of these experiments are shown in Table II. As
shown therein, the presence of a Na^+/K^+ transmembrane gradient
resulted in significantly longer drug retention times for egg-PC,
egg-PC/egg-PS (8:2), and egg-PC/cholesterol (1:1) vesicles.
Similar experiments were run with DPPC/cholesterol (1:1) vesicles.
20   In this case, complex drug release kinetics were observed, making
it difficult to determine T_{50} times. Generally, however, it
appeared that a Na^+/K^+ gradient did not significantly increase the
retention times for vesicles of this composition. The measured
data showed that in 24 hours approximately 44% of the drug was
25   released, irrespective of whether or not a transmembrane Na^+/K^+
gradient was used.

Part B

Use of pH Gradients to Reduce Drug Release Rates

Release of adriamycin from LUVs subsequent to active loading
30   using the procedures of Example 1B was assayed as follows:
vesicles (5 mM phospholipid) containing adriamycin were first
passed over a 15 ml gel filtration column equilibrated in the
appropriate buffer to remove free drug. The vesicle-containing
fraction was then placed in a flow dialysis apparatus equilibrated
35   at 37°C. Flow rates were adjusted to achieve total exchange of
the sample compartment volume (50 ml) in 10 minutes. Aliquots

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(0.15 ml) were removed at various times and untrapped material was separated employing 1 ml gel filtration columns. The sample was then assayed for adriamycin and phospholipid.

The release characteristics in the presence (panel B) and absence (panel A) of a pH gradient for EPC LUVs (circles) and EPC/cholesterol LUVs (squares) are shown in Figure 8. The solid symbols represent release characteristics in the absence of a Na^+/K^+ gradient, while the open symbols represent the release characteristics in the presence of such a gradient. Specifically, in Figure 8A adriamycin-containing vesicles were passed down desalting columns equilibrated in buffers adjusted to pH 4.6, identical to the interior pH, whereas in Figure 8B, the exterior pH was maintained at 7.5 during separation of free from vesicle-associated drug.

As shown in Figure 8, in all cases, elimination of the transmembrane pH gradient dramatically increased the rate of adriamycin efflux. Decreasing the exterior pH from 7.5 to 4.6, in the absence of Na^+/K^+ gradient (solid symbols) decreased the time for 50 percent release of trapped drug from approximately 24 to 4 hours for EPC/cholesterol LUVs (squares) and from 3 to less than 1 hour for EPC LUVs (circles). No significant effect on release kinetics was observed for EPC/cholesterol vesicles when both a pH gradient and a Na^+/K^+ gradient were used instead of just a pH gradient (compare solid and open squares). For the egg PC systems, however, the combination of a pH gradient and a Na^+/K^+ gradient resulted in longer retention times than those achieved with just a pH gradient (compare solid (pH only) and open (pH plus Na^+/K^+) circles). This effect was most pronounced for EPC LUVs in the presence of the transmembrane pH gradient (panel B), where the time for 50 percent adriamycin release increased from 3 to 16 hours when a Na^+/K^+ gradient was superimposed on the pH gradient. Note that a 16 hour T_{50} value was also obtained for EPC vesicles using just a Na^+/K^+ gradient (see Table II).

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Example 3
Dehydration of Liposomes
Containing an Antineoplastic Agent

This example illustrates the dehydration and subsequent
rehydration of liposomes containing the antineoplastic agent
adriamycin.

Egg phosphatidylcholine ETVs were prepared as described above
using a solute solution (169 mM KGlu, 20 mM HEPES (pH 7.4), 40
umol lipid/ml) containing 250 mM trehalose. Subsequently, the
external potassium buffer was exchanged for a sodium buffer (150
mM NaCl, 20 mM HEPES (pH 7.4), 250 mM trehalose). Adriamycin (200
nmol/umol lipid) was added, along with valinomycin (0.5 ug/umol
lipid) to induce the membrane potential. After a 2 hour
incubation, unencapsulated adriamycin was removed by passing the
vesicles through a column of Sephadex G-50 equilibrated with the
trehalose-containing sodium buffer described above. The ETVs were
dehydrated for 24 hours without prior freezing and then rehydrated
as described above.

The amounts of entrapped adriamycin in the vesicles both
before and after dehydration/rehydration, as well as the rate of
drug leakage from the vesicles, were measured using the assay
described above (see "Assays") after passage of 100 ul aliquots of
the vesicle suspension over columns (1 ml) of Sephadex G-50 to
remove any untrapped material (see U.S. Patent Application Serial
No. 622,690 for further details). Since the columns tend to trap
a small percentage of the liposomes applied thereto, the measured
values for the amounts of encapsulated material retained after the
dehydration/rehydration process are somewhat lower than the levels
actually achieved by the procedures of the present invention.

The results of these experiments are shown in Table III. As
shown therein, more than 90% of the drug is retained following
dehydration and rehydration. Moreover, the rate of leakage of
adriamycin from the rehydrated vesicles is comparable to the rate
observed with vesicles which have not been dehydrated (data not
shown).

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Example 4
Loading of Ionizable Antineoplastic Agents Into
Rehydrated Liposomes Using Transmembrane Potentals

This example illustrates: 1) that liposomes having a
concentration gradient across their membranes can be dehydrated in
the presence of a protective sugar and rehydrated without loss of
the concentration gradient; and 2) that after rehydration, the
concentration gradient can be used to load an ionizable
antineoplastic agent (adriamycin) into the liposomes.

Vesicles having a Na^+/K^+ chemical gradient across their
membranes were prepared by forming ETVs (40 umol lipid/ml) in a
potassium glutamate buffer (169 mM potassium glutamate, 250 mM
trehalose, 20 mM HEPES, pH 7.4), and then replacing the external
buffer with a NaCl buffer (150 mM NaCl, 250 mM trehalose, 20 mM
HEPES, pH 7.4) by passing the vesicles through a Sephadex G-50
column (1.4 x 10 cm) which had been pre-equilibrated with the NaCl
solution. Where employed, valinomycin (Sigma, St. Louis,
Missouri) was added in ethanol to a concentration of 0.5 ug/umole
phospholipid.

Similarly, transmembrane pH gradients (interior acid) were
formed by preparing the liposomes in a buffer with low pH (135 mM
glutamic acid, 250 mM trehalose, brought to pH 5.5 by the addition
of potassium hydroxide) which was then exchanged with a high pH
buffer (125 mM glutamic acid, 30 mM NaCl, 250 mM trehalose,
brought to pH 7.5 by the addition of potassium hydroxide) on a
Sephadex G-50 column. Where used, the proton ionophore CCCP was
added to a final concentration of 20 uM.

Transmembrane potentials were measured by determining the
distribution of the lipophilic cation ^{3}H-tetraphenylphosphonium
bromide (^{3}H-TPPB, NEN, Canada). Specifically, 1 uCi of ^{3}H-TPPB in
1 ul ethanol was added to a 1-2 ml sample of the ETV dispersion
and the mixture was incubated at 20°C for 20 minutes. An aliquot
(100 ul) was withdrawn and the untrapped ^{3}H-TPP$^+$ was removed by
loading the aliquot onto a Sephadex G-50 column packed in a 1 ml
disposable syringe, and then centrifuging the column at 500 g for
3 minutes to elute the vesicles. The trapped ^{3}H-TPP$^+$ was

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determined by liquid scintillation counting, and the phospholipid determined by phosphate assay.

Using trapped volume values (ul per umol of phospholipid) for the ETVs determined by measuring the amount of ^{22}Na, ^{3}H-inulin, or ^{14}C-inulin captured in the ETVs by the ETV process, the concentrations of ^{3}H-TPP^{+} inside $[^{3}H$-TPP$^{+}]_i$ and outside $[^{3}H$-TPP$^{+}]_o$ the vesicles were calculated, from which the transmembrane potential (V_m) was calculated using the Nernst equation:

$$V_m = -59 \log [^{3}H\text{-}TPP^{+}]_i/[^{3}H\text{-}TPP^{+}]_o.$$

Both the Na^{+}/K^{+} and the pH gradient vesicles were dehydrated under high vacuum for 24 hours and then rehydrated. Control vesicles were kept at 4°C for 24 hours. Following drying and rehydration, the transmembrane potentials exhibited by these vesicles in the presence and absence of ionophores were compared to the transmembrane potentials generated by the controls, also in the presence and absence of ionophores. The results are shown in Figures 9 (pH) and 10 (Na^{+}/K^{+}).

As can be seen from these figures, the transmembrane potentials exhibited by the vesicles which had been dehydrated and then rehydrated are essentially identical to those exhibited by the controls. The only apparent difference is that in the case of the pH gradient vesicles, the transmembrane potentials for the dehydrated/rehydrated vesicles develop somewhat slower than the transmembrane potentials for the control vesicles.

The ability of the Na^{+}/K^{+} vesicles to accumulate adriamycin after dehydration and rehydration was tested in the presence and absence of the ionophore valinomycin, and compared with the accumulation exhibited by the control vesicles, i.e., the vesicles which had been stored at 4°C for 24 hours rather than being dehydrated for 24 hours. Sufficient adriamycin was added to the vesicles' external medium to produce a final concentration of 0.2 moles adriamycin per mole of phospholipid.

The results of these tests are shown in Figure 11. As can be seen therein, the dehydrated/rehydrated vesicles accumulate adriamycin essentially at the same rate and to the same extent as the control vesicles. Accordingly, this example demonstrates that

delayed loading of vesicles can be accomplished through the combination of concentration gradients and the dehydration/rehydration process.

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TABLE I

ACTIVE/PASSIVE TRAPPING
OF ANTINEOPLASTIC AGENTS IN LIPOSOMES

Drug	Trapping Efficiency (%)	nmol drug per umol lipid
Methotrexate	33	35.3
+ adriamycin	99	99
Cytosine arabinoside	33	44.5
+ adriamycin	98	98
Vinblastine	90	36
Adriamycin	95	95

Methotrexate was trapped passively at a concentration of 20 mM during preparation of vesicles (187 umol lipid/ml). Adriamycin was trapped employing a transmembrane Na^+/K^+ gradient in the presence of valinomycin with a vesicle concentration corresponding to 1 mM phospholipid and a starting adriamycin concentration of 100 uM. Cytosine arabinoside was trapped passively, at a concentration of 25 mM, during preparation of vesicles (187 umol lipid/ml), and vinblastine was trapped employing a transmembrane Na^+/K^+ gradient in the presence of valinomycin with a vesicle concentration corresponding to 5 mM phospholipid and a starting vinblastine concentration of 200 uM.

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Table II

DRUG RELEASE FROM LUVs IN THE PRESENCE AND ABSENCE OF A TRANSMEMBRANE POTENTIAL

Lipid Composition	Agent Trapped	External Buffer	$T_{50}(h)$
Egg-PC	adriamycin	KCl	1.5
		NaCl	16
Egg-PC/egg-PS(8:2)	adriamycin	KCl	1.5
		NaCl	6
Egg-PC/Chol(1:1)	adriamycin	KCl	4
		NaCl	30

After drug encapsulation the external buffer and untrapped drug were replaced with either a KCl or NaCl buffer as specified. T_{50} indicates the time needed for release of 50% of the trapped agent from the vesicles; Chol = cholesterol.

TABLE III

ABILITY OF DEHYDRATED VESICLES TO RETAIN ADRIAMYCIN

	Adriamycin Content (nmoles/umole lipid)
Before dehydration	197
Immediately after dehydration and rehydration	185
One hour after dehydration and rehydration	158
Two hours after dehydration and rehydration	145

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What is claimed is:

1.   A method for loading liposomes with an ionizable antineoplastic agent comprising the steps of:

(a) preparing liposomes having a concentration gradient of one or more charged species across their membranes, said concentration gradient being capable of generating a transmembrane potential having an orientation which will cause the ionizable antineoplastic agent to be loaded into the liposomes; and

(b) admixing the ionizable antineoplastic agent with the liposomes.

2.   The method of Claim 1 wherein the concentration gradient is produced by:

(a) encapsulating a first medium in the liposomes, said medium having a first concentration of the one or more charged species; and

(b) suspending the liposomes in a second medium having a second concentration of the one or more charged species.

3.   The method of Claim 1 wherein the concentration gradient is a Na^+/K^+ concentration gradient.

4.   The method of Claim 1 wherein the concentration gradient is a pH gradient.

5.   The method of Claim 1 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

6.   A pharmaceutical preparation comprising an ionizable antineoplastic agent which has been loaded into liposomes by the method of Claim 1.

7.   The pharmaceutical preparation of Claim 6 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

8.   A pharmaceutical preparation comprising dehydrated liposomes having encapsulated therein an antineoplastic agent.

9.   The pharmaceutical preparation of Claim 8 wherein the antineoplastic agent is selected from the group consisting of

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daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

10. A method for loading liposomes with an ionizable antineoplastic agent comprising the steps of:

(a) preparing liposomes having a concentration gradient of one or more charged species across their membranes, said concentration gradient being capable of generating a transmembrane potential having an orientation which will load the ionizable antineoplastic agent into the liposomes;

(b) dehydrating the liposomes;

(c) storing the dehydrated liposomes;

(d) rehydrating the dehydrated liposomes; and

(e) admixing the ionizable antineoplastic agent with the rehydrated liposomes.

11. The method of Claim 10 wherein the concentration gradient is produced by:

(a) encapsulating a first medium in the liposomes, said medium having a first concentration of the one or more charged species; and

(b) suspending the liposomes in a second medium having a second concentration of the one or more charged species.

12. The method of Claim 11 wherein the concentration gradient is a Na^+/K^+ concentration gradient.

13. The method of Claim 11 wherein the concentration gradient is a pH gradient.

14. The method of Claim 10 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

15. A pharmaceutical preparation comprising an ionizable antineoplastic agent which has been loaded into liposomes by the method of Claim 10.

16. The pharmaceutical preparation of Claim 15 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

17. A method for loading liposomes with an ionizable antineoplastic agent comprising the steps of:

(a) preparing a liposome preparation;

(b) dehydrating the liposome preparation;

(c) storing the dehydrated preparation;

(d) rehydrating the dehydrated preparation;

(e) replacing the external medium surrounding the liposomes in the rehydrated preparation with a medium which will produce a concentration gradient of one or more charged species across the membranes of the liposomes, said concentration gradient being capable of generating a transmembrane potential having an orientation which will load the ionizable antineoplastic agent into the liposomes; and

(f) admixing the ionizable antineoplastic agent with the liposomes in their replaced external medium.

18. The method of Claim 17 wherein the concentration gradient is a Na^+/K^+ concentration gradient.

19. The method of Claim 17 wherein the concentration gradient is a pH gradient.

20. The method of Claim 17 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmacuetically acceptable salts and derivatives thereof.

21. A pharmaceutical preparation comprising an ionizable antineoplastic agent which has been loaded into liposomes by the method of Claim 17.

22. The pharmaceutical preparation of Claim 21 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

23. A method for reducing the rate of release of an ionizable, biologically-active agent from liposomes comprising generating a transmembrane potential across the liposome membranes which has an orientation such that if the agent is positively charged, the internal potential of the liposomes is negative relative to the potential of the external medium, and if the agent

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is negatively charged, the internal potential of the liposomes is positive relative to the potential of the external medium.

24. The method of Claim 23 wherein the transmembrane potential is generated by creating a concentration gradient of one 5   or more charged species across the liposome membranes.

25. The method of Claim 24 wherein the concentration gradient is a Na^+/K^+ concentration gradient.

26. The method of Claim 24 wherein the concentration gradient is a pH gradient.

10   27. The method of Claim 23 wherein the agent is an antineoplastic agent.

28. The method of Claim 27 wherein the antineoplastic agent is selected from the group consisting of daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts 15   -and derivatives thereof.

29. A pharmaceutical preparation comprising liposomes having encapsulated therein an ionizable, biologically-active agent, said liposomes having a transmembrane potential across their membranes, the orientation of the transmembrane potential being 20   such such that if the agent is positively charged, the internal potential of the liposomes is negative relative to the potential of the external medium, and if the agent is negatively charged, the internal potential of the liposomes is positive relative to the potential of the external medium.

25   30. The pharmaceutical preparation of Claim 29 wherein the transmembrane potential has been produced by creating a concentration gradient of one or more charged species across the liposome membranes.

31. The pharmaceutical preparation of Claim 30 wherein the 30   concentration gradient is a Na^+/K^+ concentration gradient.

32. The pharmaceutical preparation of Claim 30 wherein the concentration gradient is a pH gradient.

33. The pharmaceutical preparation of Claim 29 wherein the agent is an antineoplastic agent.

35   34. The pharmaceutical preparation of Claim 33 wherein the antineoplastic agent is selected from the group consisting of

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daunorubicin, doxorubicin, vinblastine, and pharmaceutically acceptable salts and derivatives thereof.

35. A pharmaceutical preparation comprising liposomes having two or more antineoplastic agents encapsulated therein.

36. The pharmaceutical preparation of Claim 35 wherein one of the antineoplastic agents is an ionizable antineoplastic agent.

37. The pharmaceutical preparation of Claim 36 wherein the ionizable antineoplastic agent has been loaded into the liposomes by means of a transmembrane potential.

38. The pharmaceutical preparation of Claim 37 wherein the ionizable antineoplastic agent is doxorubicin.

39. The pharmaceutical preparation of Claim 35 wherein the two or more antineoplastic agents include doxorubicin and methotrexate.

40. The pharmaceutical preparation of Claim 35 wherein the two or more antineoplastic agents include doxorubicin and cytosine arabinoside.

41. A method of treating neoplasms comprising administering an antineoplastic effective amount of the pharmaceutical preparation of Claim 6.

42. A method of treating neoplasms comprising rehydrating the pharmaceutical preparation of Claim 8 and administering an antineoplastic effective amount of the rehydrated preparation.

43. A method of treating neoplasms comprising administering an antineoplastic effective amount of the pharmaceutical preparation of Claim 15.

44. A method of treating neoplasms comprising administering an antineoplastic effective amount of the pharmaceutical preparation of Claim 21.

45. A method of treating neoplasms comprising administering an antineoplastic effective amount of the pharmaceutical preparation of Claim 33.

46. A method of treating neoplasms comprising administering an antineoplastic effective amount of the pharmaceutical preparation of Claim 35.

FIG.1

FIG. 2

FIG.3

FIG.4B

FIG.4A

n mole ADM/μmole lipid

Time(minutes)
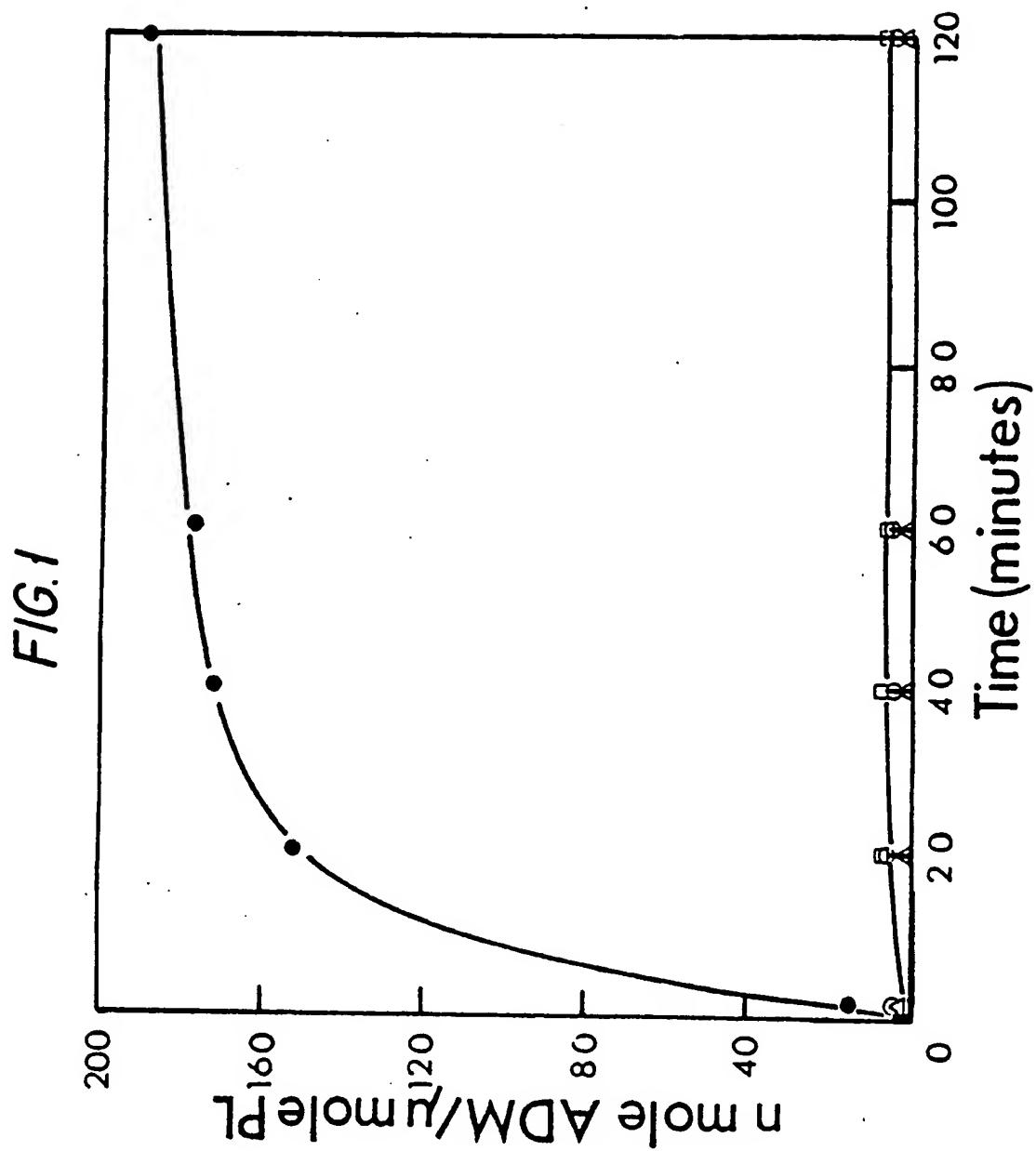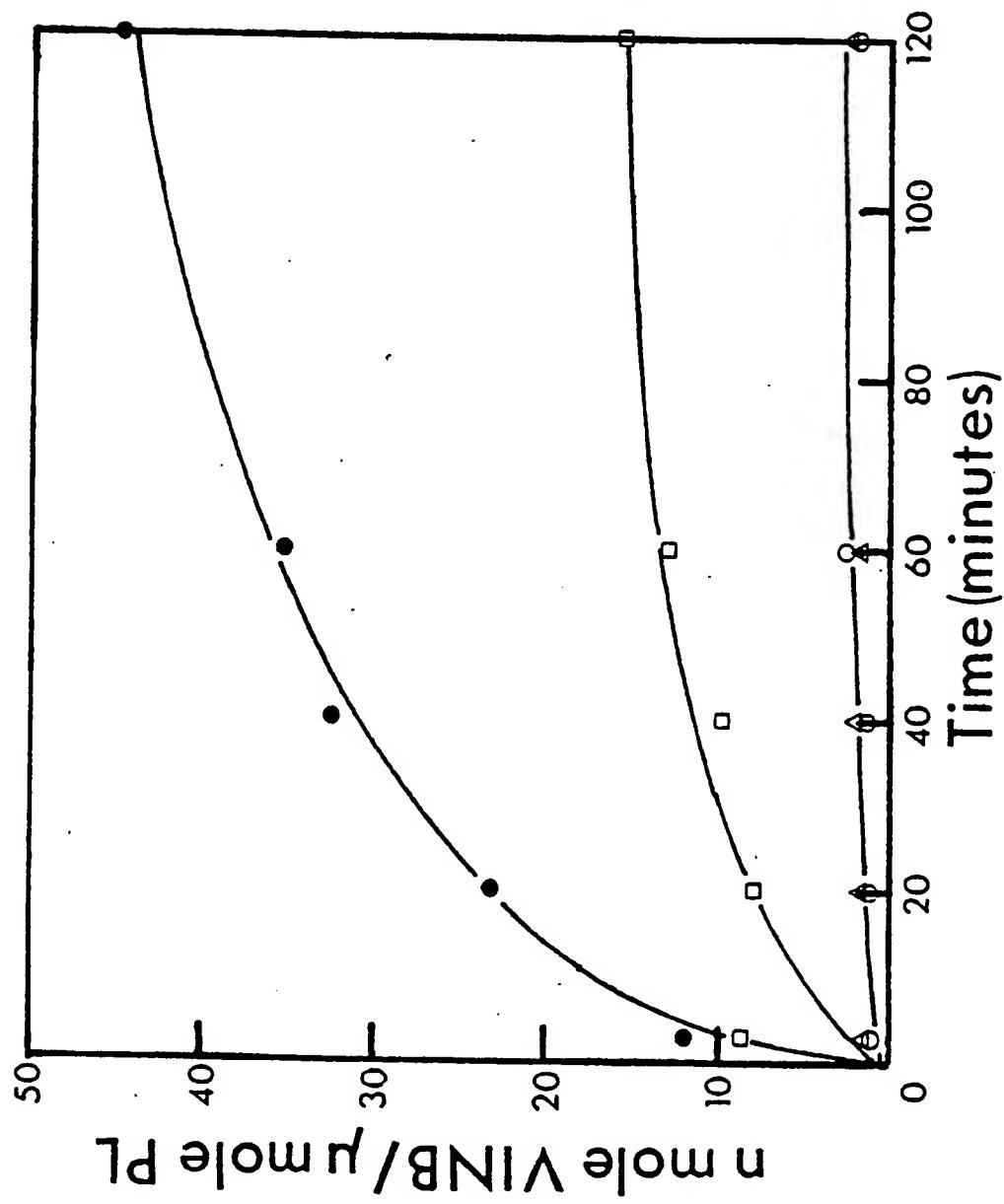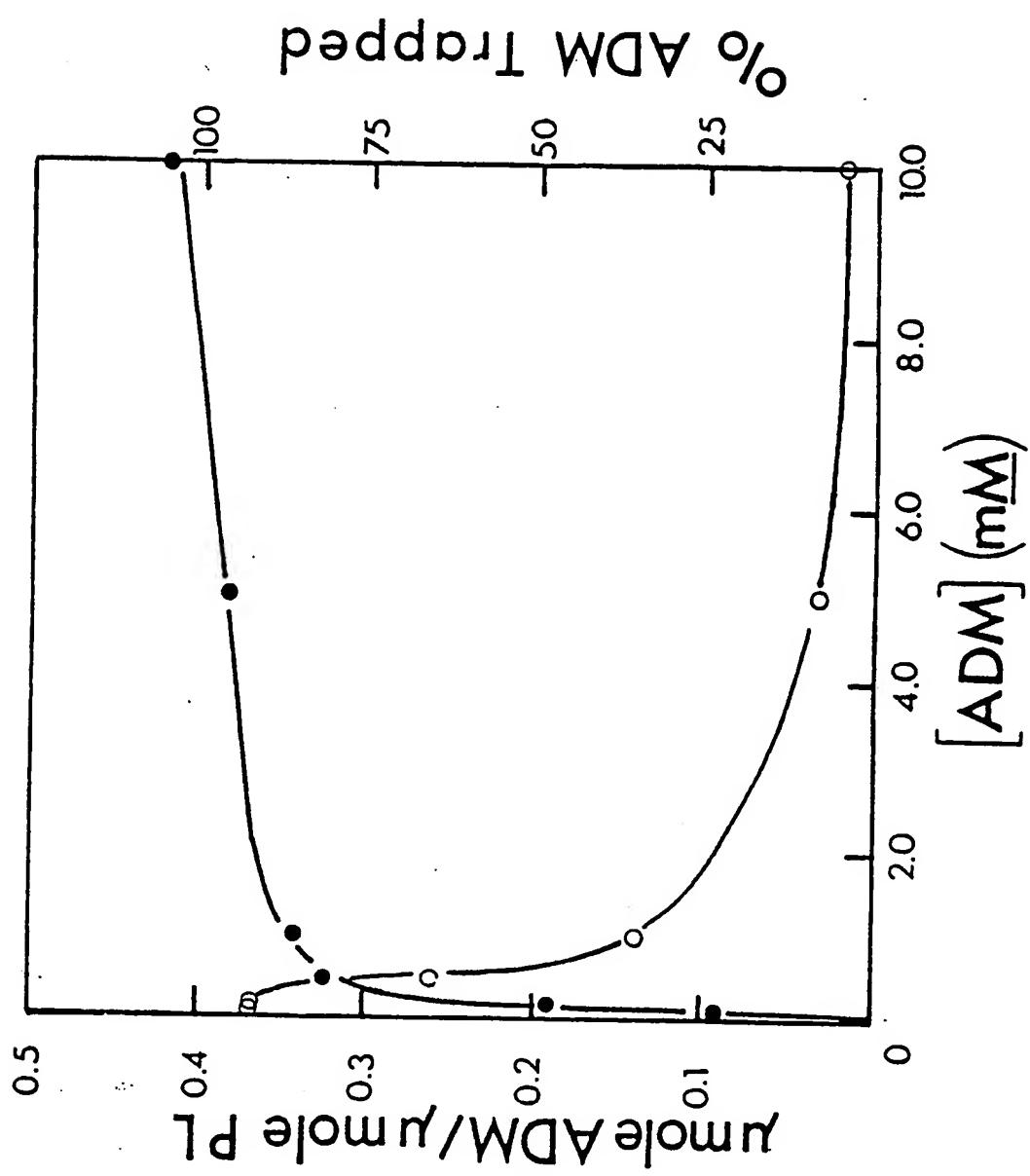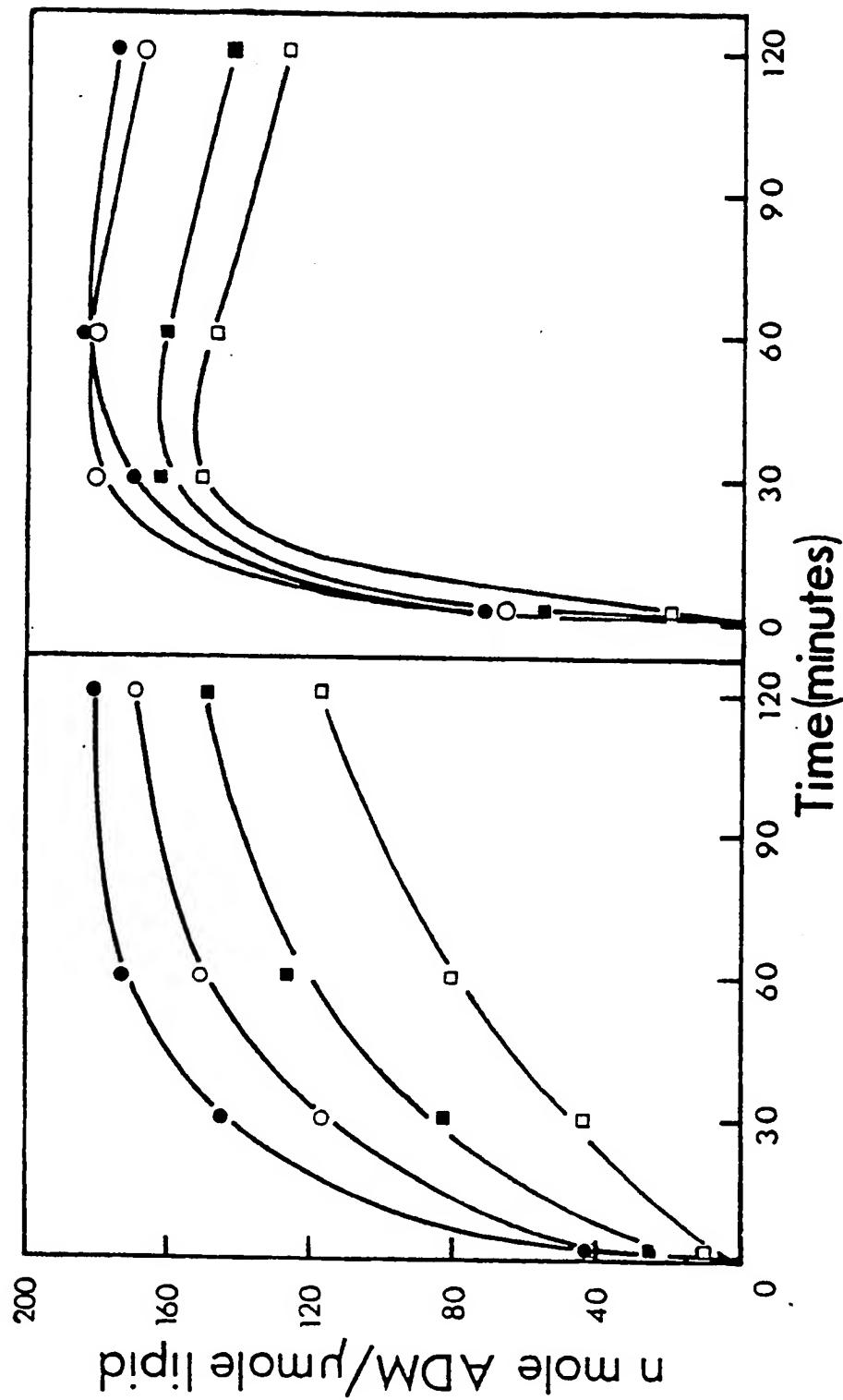
FIG. 5
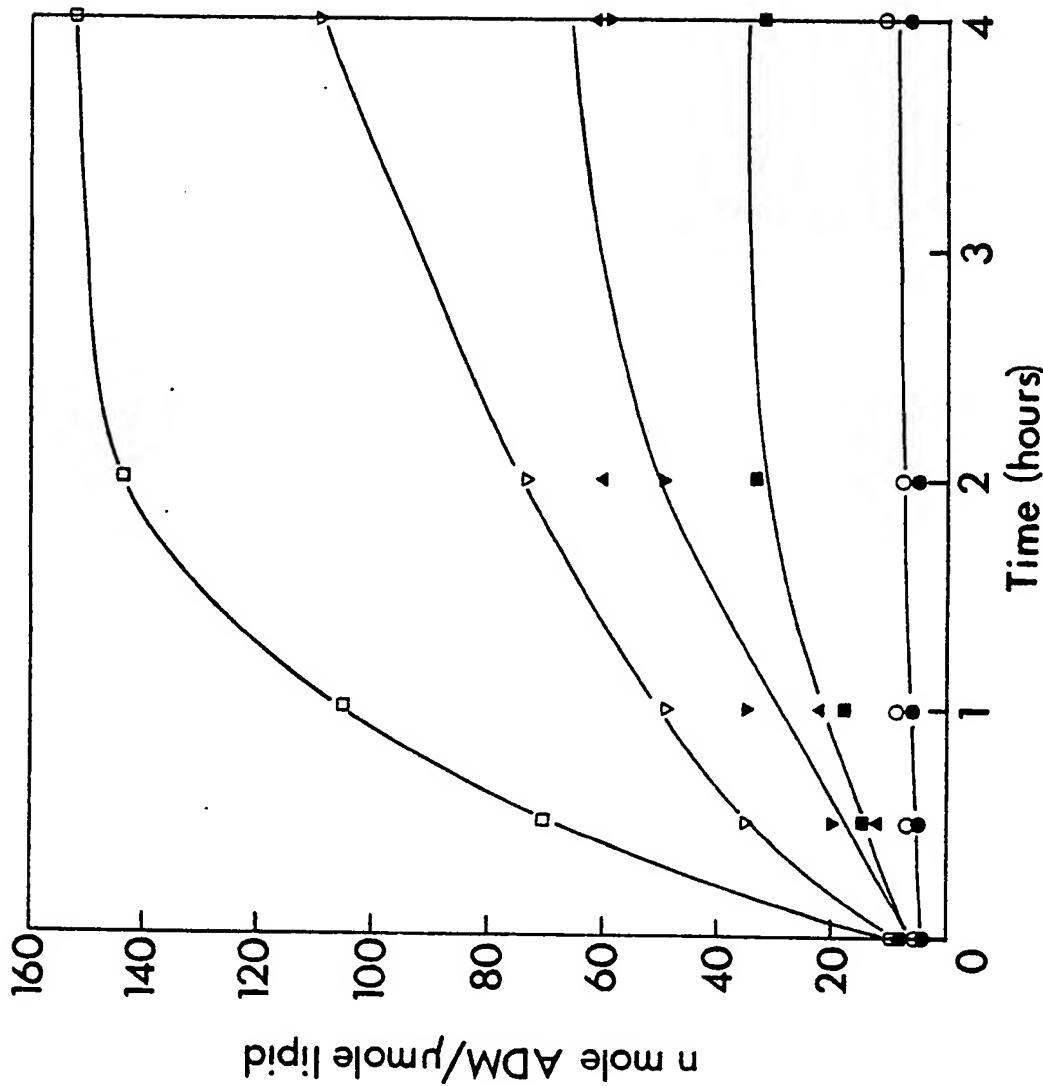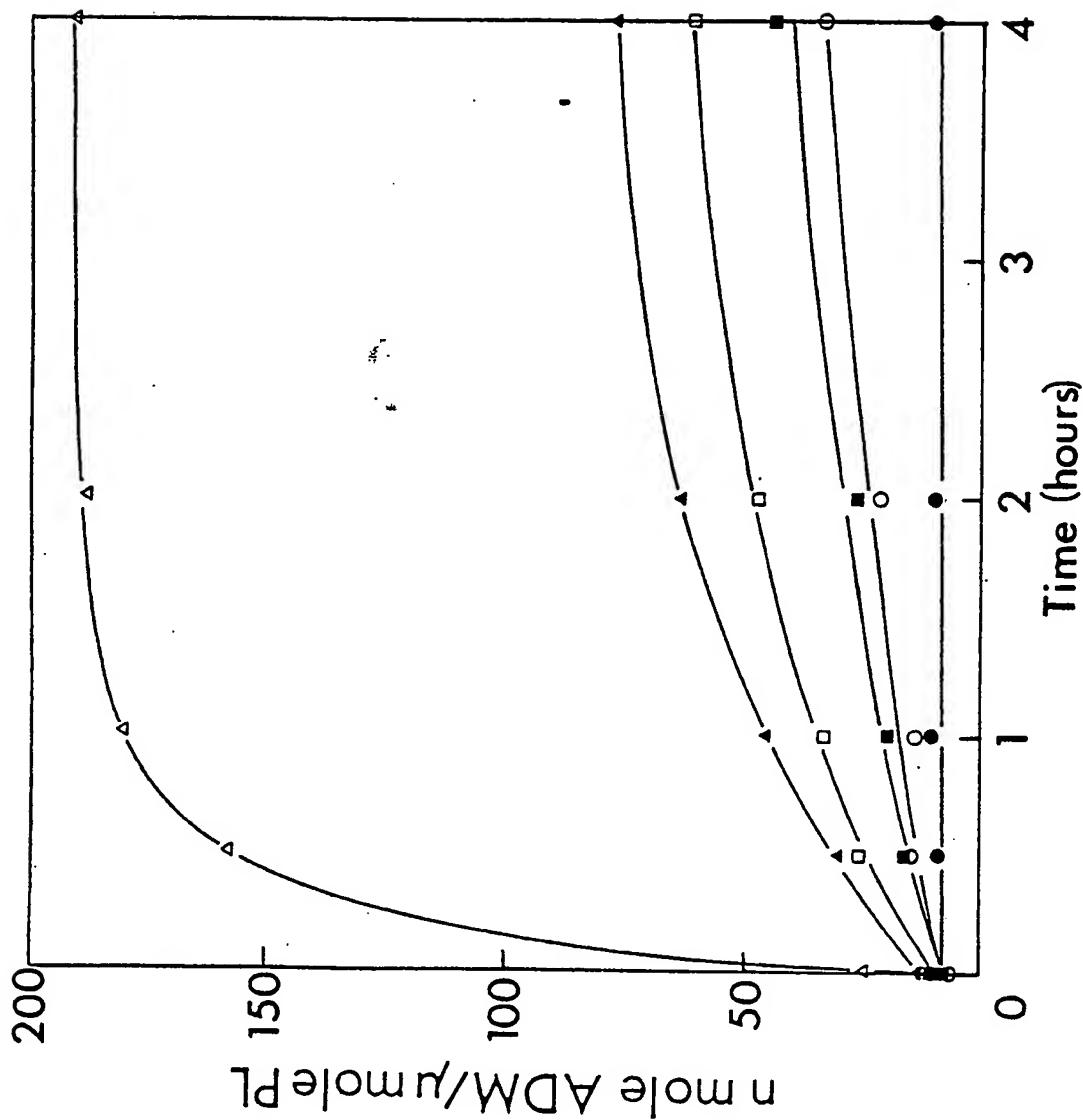
FIG.6
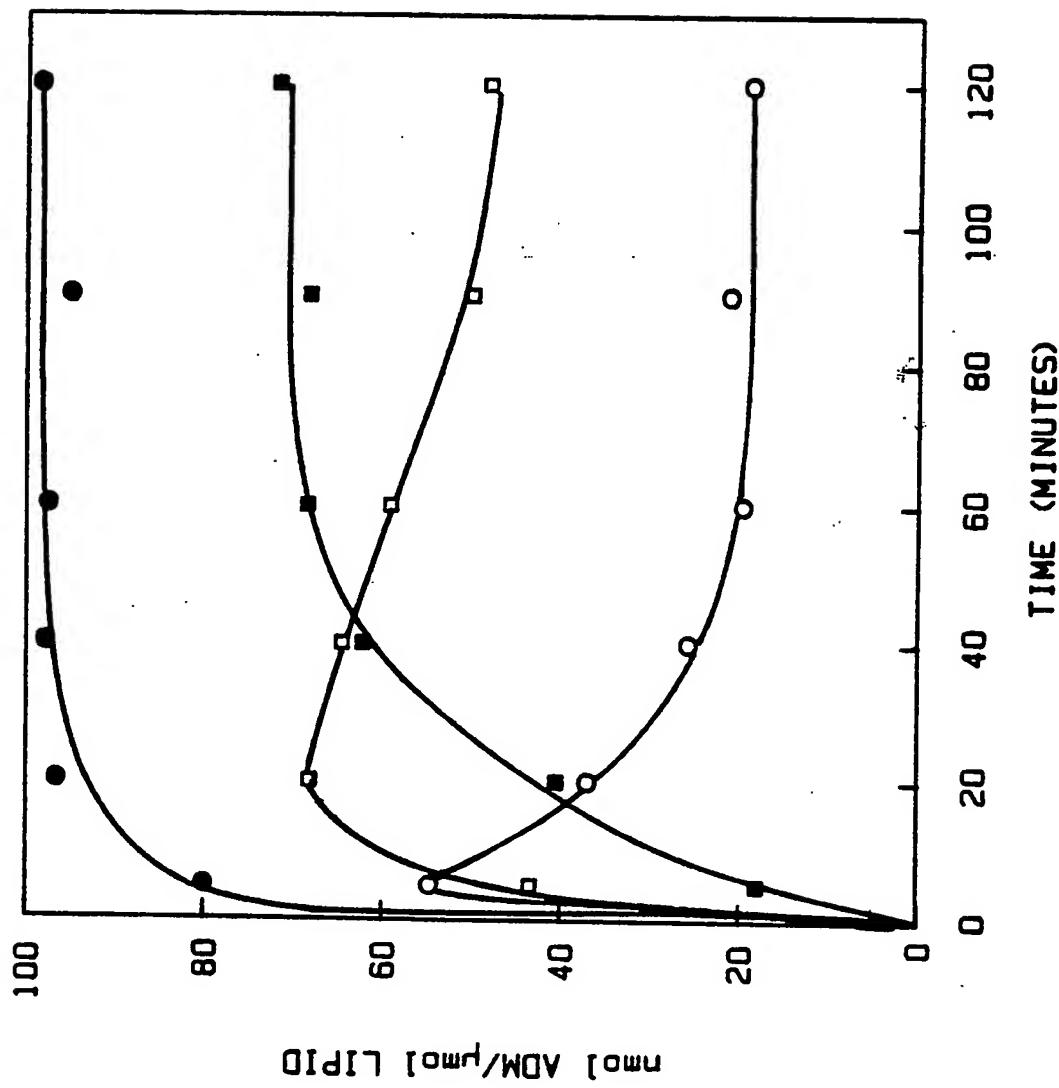
FIG. 7A
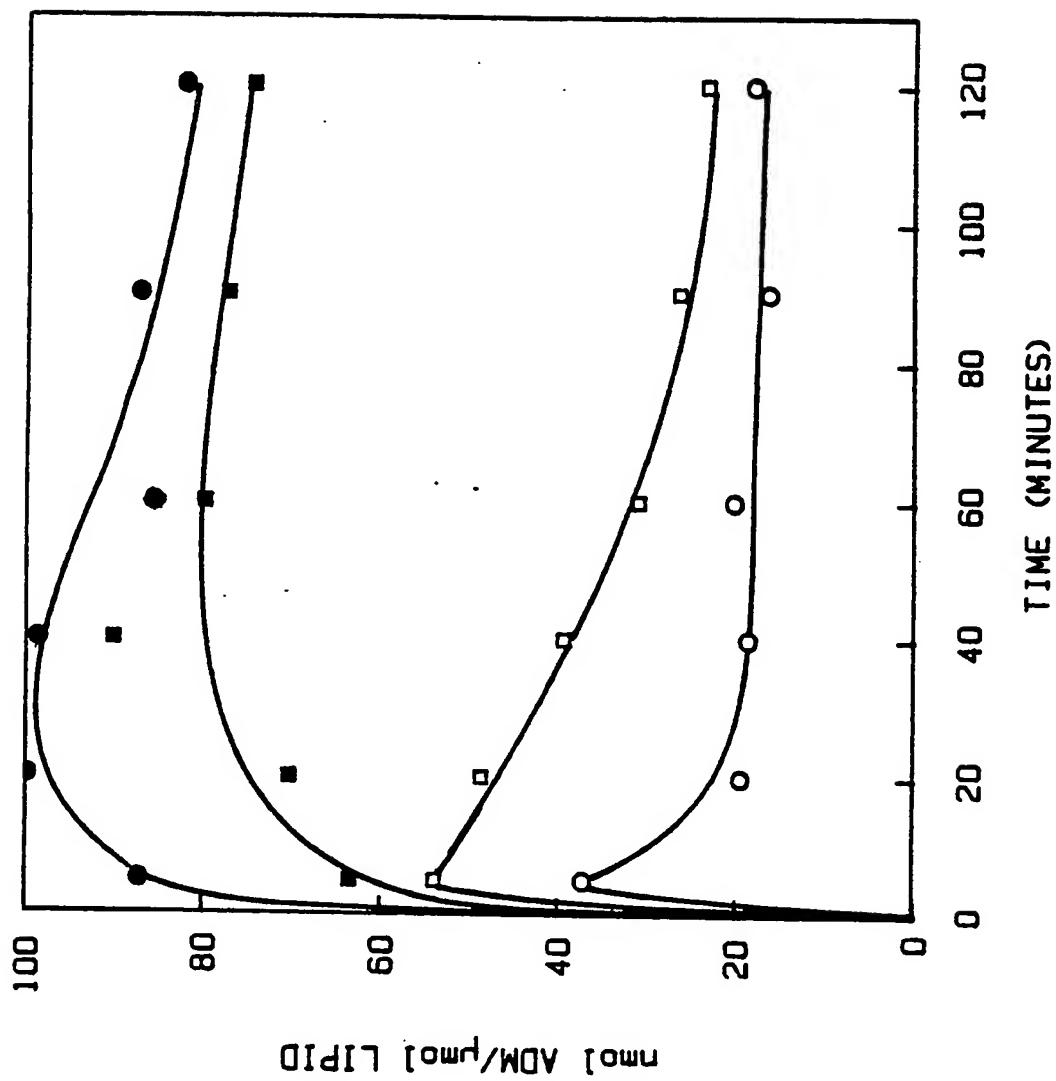
FIG.7B

FIG. 8A
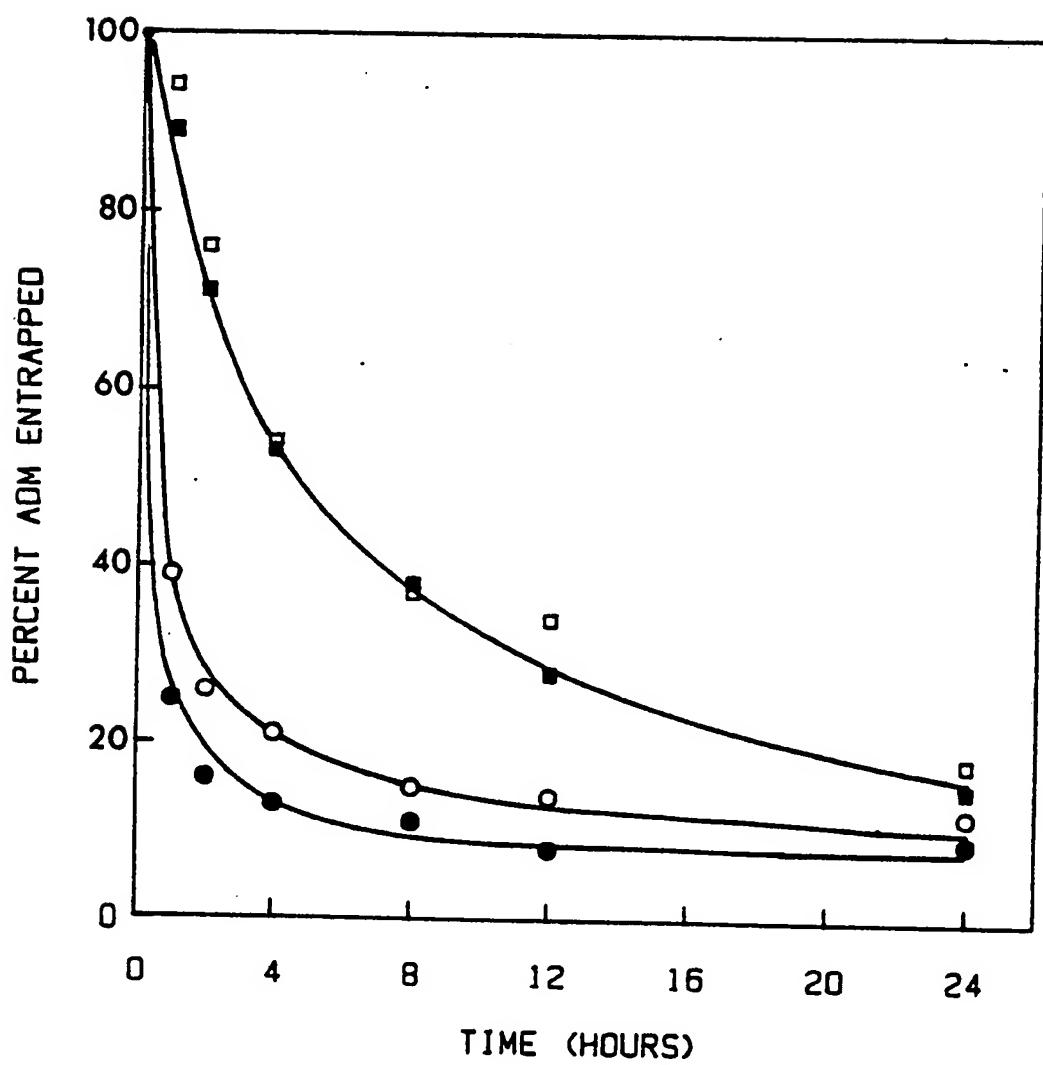
FIG.8B
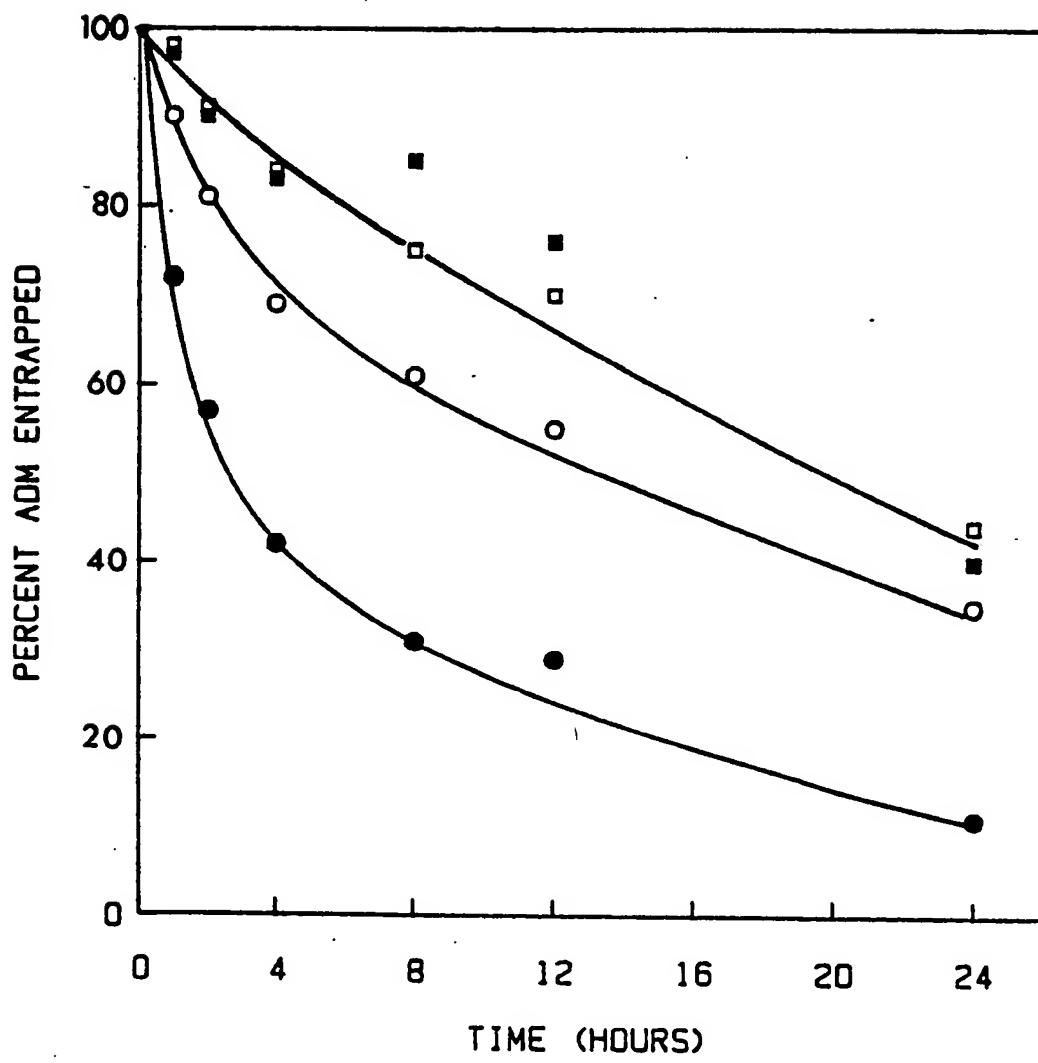
FIG.9
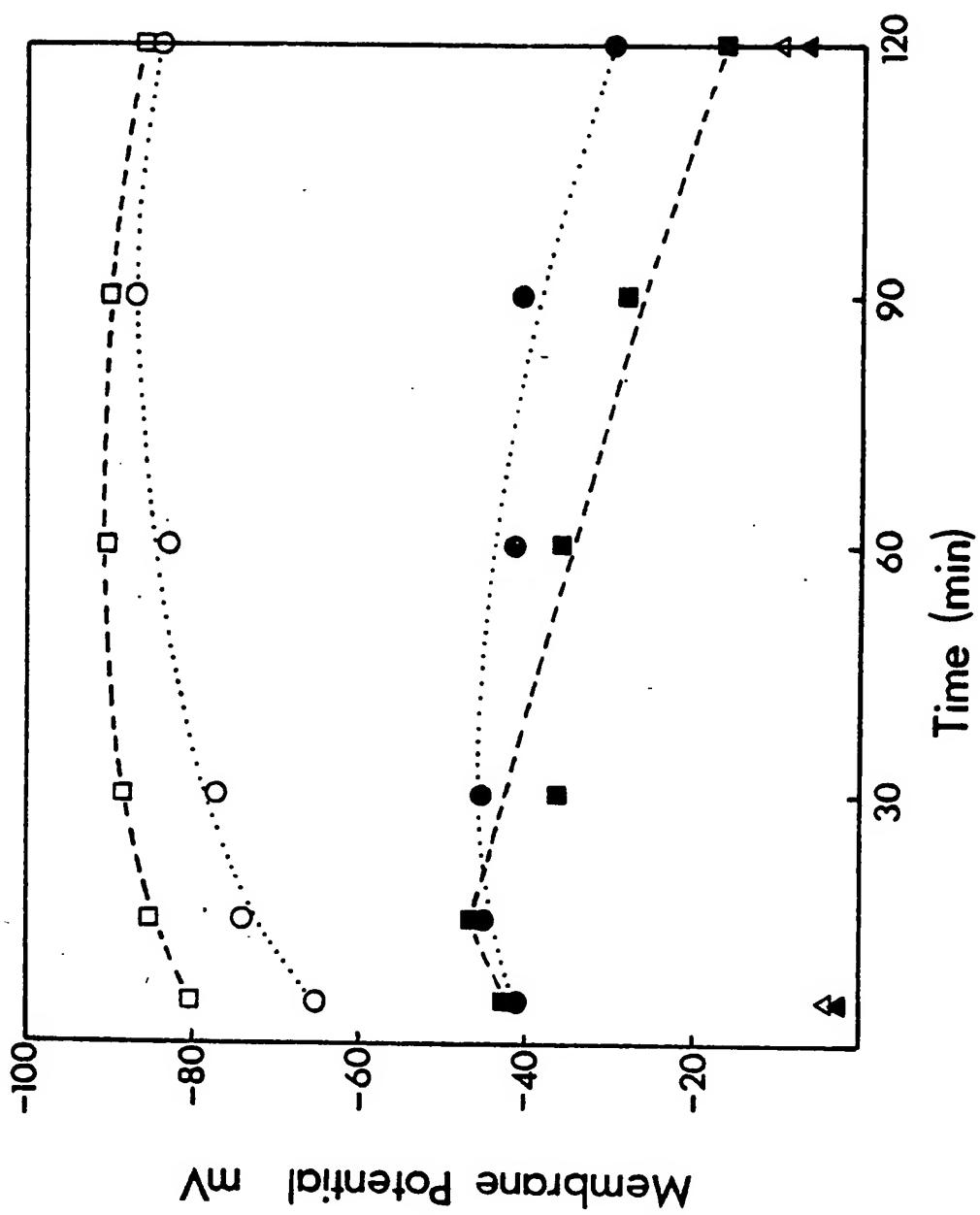
FIG. 10
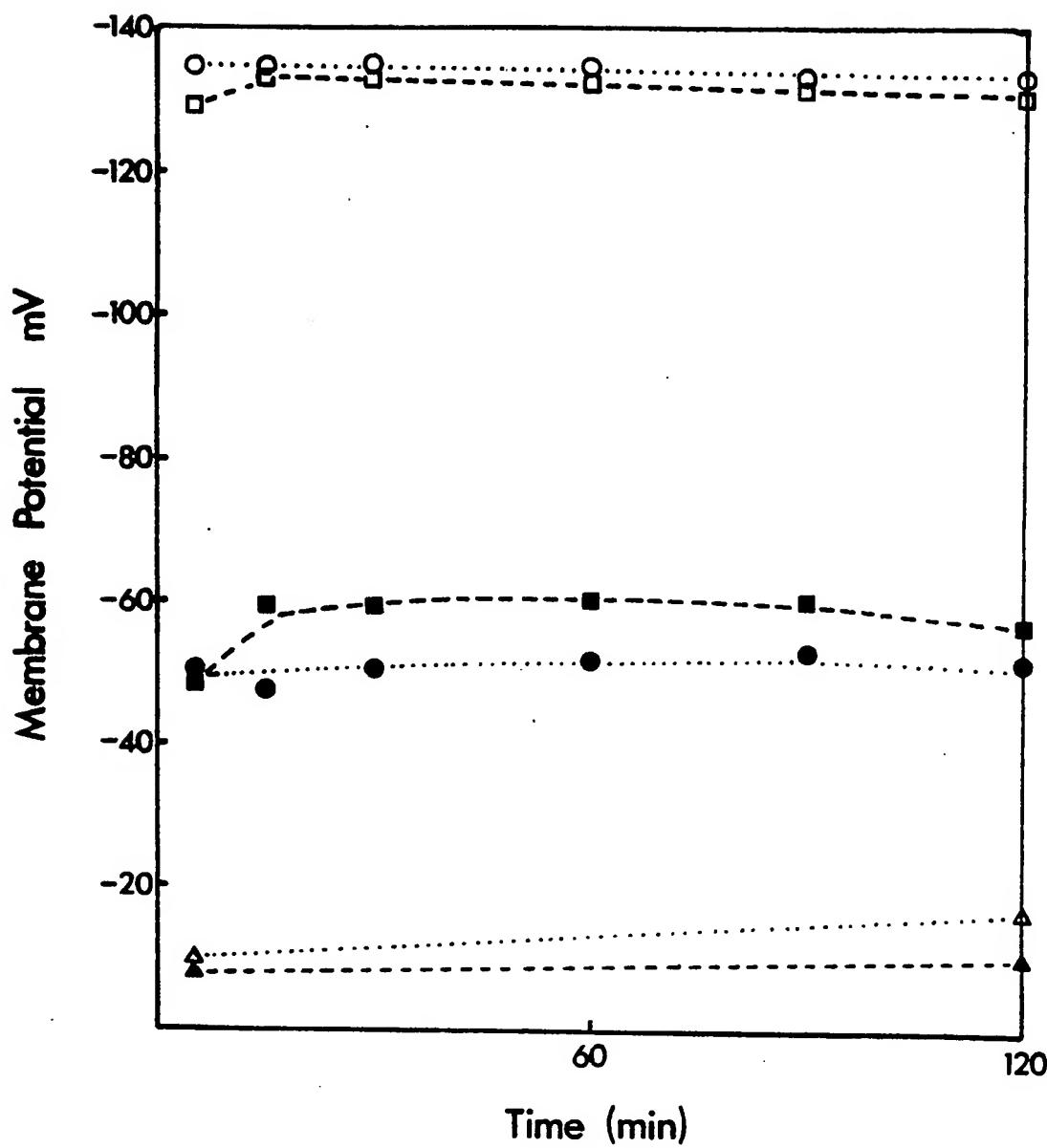
13 / 13

FIG. 11
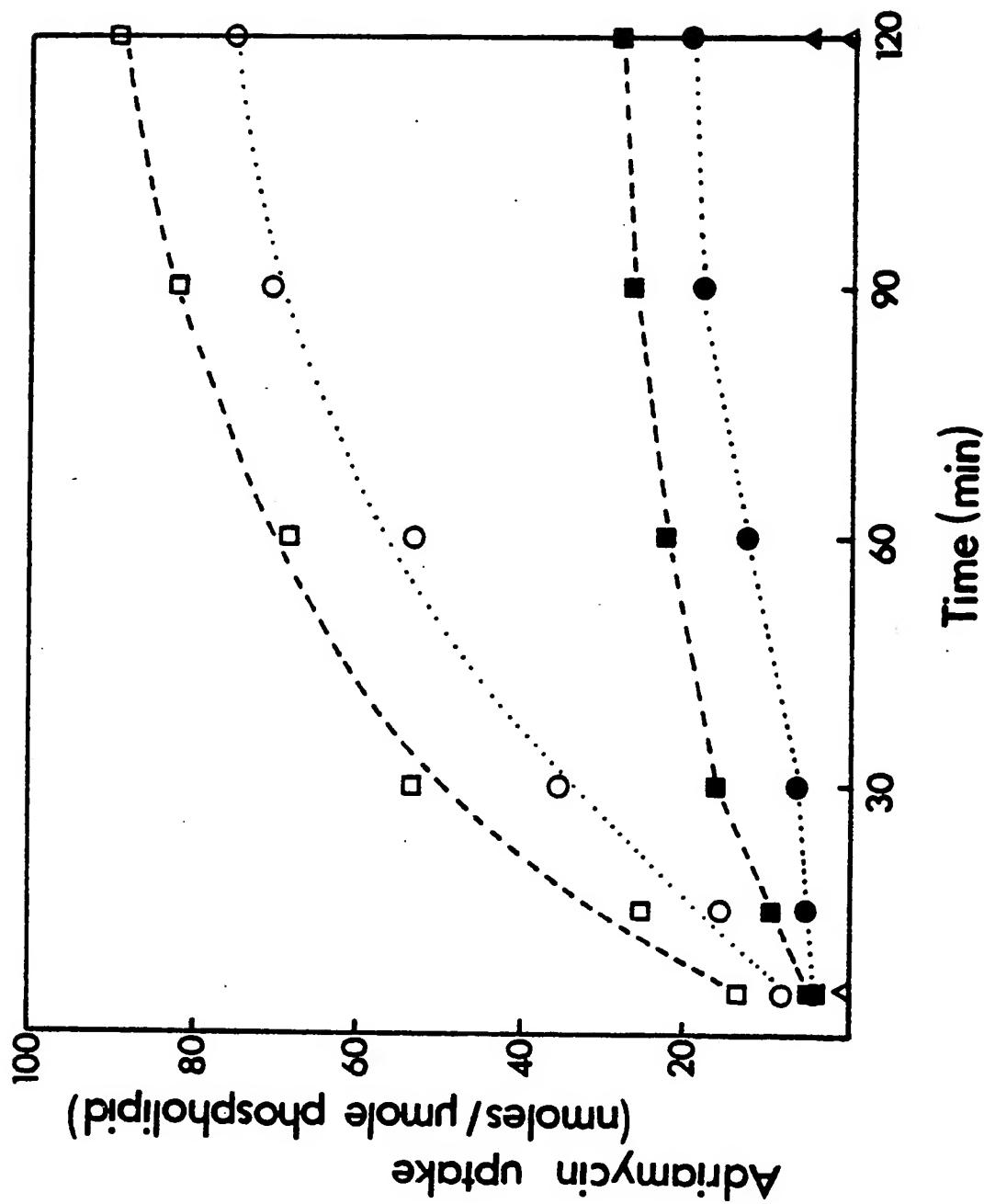
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC   4th Edition

A61K 9/60, A61K 9/52, A61K 7/44, A61K 39/00, B01J 13/02

II. FIELDS SEARCHED

Minimum Documentation Searched 4	
Classification System	Classification Symbols
U.S.	424/19,38 514/943 264/4.3,4.6,4.7

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	US,A, 4,193,983, Ullman et al., published 18 May 1980	1-46
Y	N,Biological Abstracts, Vol. 77(7), 1984, Garcia et al., "Mechanism of lactose translocation in proteoliposomes reconstituted with lac carrier protein purified from E. coli: 1. Effect of pH and imposed membrane potential on efflux, exchange and counter flow". See page 6013, column 1, Abstract No. 54638, Biochemistry 22(10): 2524-2531, 1983	1-46
Y	N,Biological Abstracts 77(12), 1984 Shakhov et al, "Reconstitution of highly purified proton-trans-locating pyrophosphate from Rhocuspirillium rubrum., See page 10459 column 1, Abstract No. 94506, Biokhimika 48(8): 1347-1351.	1-46

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 3	Date of Mailing of this International Search Report 3
22 Oct 1985	3 1 OCT 1985
International Searching Authority 1	Signature of Authorized Officer 20
IS/US	

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
X.Y	N.Chemical Abstracts. Vol. 94. 1981 (Columbus Ohio) Moro et al. "Purification of liposome suspensions" See page 372. column 1. Abstract No 52931a. Ger. Offen 3.001.842. 31 July 1980	1-46
X.Y	N.Chemical Abstracts. Vol. 102. 1985 (Columbus. Ohio) Kirby et al "Dehydration-rehydration vesicles: A simple method for high yield drug entrapment in liposomes" Column 1. Abstract No. 84326w. Bio/Techmology . 1984 2(11). 979-84.	1-46
Y	N. Chemical Abstracts. Vol. 99. 1983 (Columbus. Ohio) Crommelin et al. "Preparation and Characterization of doxorubicin containing liposomes I. Influence of liposome charge and pH of hydration medium on loading capacity and particle size". Column 1 Abstract No 128259c. Int. J. Pharm 16(1) 79-92 (1983).	1-46
Y	N.Chemical Abstracts. Vol. 102. 1985. (Columbus. Ohio) Groom et al "Liposomes" page 337. Column 1. Abstract No 67398d Brit UK Pat. Appli. GB 2.134.869. 22 Aug 1984	1-46

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
Y	N,Chemical Abstracts. Vol. 101. 1984 Van Hoesel et al "Reduced cardiotoxicity and nephrotoxicity with preservation of antitumor activity of doxorubicin entrapped in stable Liposomes in the LOU/M Wsl rat.". page 24. column 1. Abstract No 163307d: Cancer Res 44(9) 3698-705. 1984	1-46
Y	N,Chemical Abstracts. Vol. 101. 1984 Lenk et al "Stabilized purieamellar vesicles: A new type of liposome". page 62. column 1. Abstract 83980X Braz. Pedido PI BR 83. 06. 785. 7 March 1984.	1-46
Y	N,Chemical Abstracts. Vol. 100. 1984 Lenk et al "Stable plurilamellar vesicles page 347. column 1. Abs. 215560b. S. African 28 83 02.197. 28 Dec 1983	1-46
Y	N,Chemical Abstracts. Vol. 100. 1984 Crommelin et al "Preparation and Characterization of doxorubicin-containing liposomes II. capacity. long term stability and doxorubicin-bilayer interaction mechanism. page 349. column 1. Abst. 109032w. Int.J. Pharm 1983 17(2-3) 135-44.	1-46
Y	US.A. 4.438.052. Weder et al. published 20 March 1984	1-46

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, [16] with indication, where appropriate. of the relevant passages [17]	Relevant to Claim No [18]
X,Y	US,A, 4,411,894, Schrank et al published 25 Oct, 1983	1-46
X,Y	US,A, 3,962,429, Furuno et al, published 8 June 1976	1-46
P,X,Y	US,A, 4,515,736, Deamer, published 7 May 1985	1-46
Y	US,A, 4,217,344, Van der berghe et al published 12 Aug. 1980	1-46
Y	US,A, 4,389,330 Tice et al, published 21 June 1983	1-46
A	US,A, 4,235,871, Regen, 25 Nov 1984	
A	US,A, 4,241,046, Papahadjopoulos, 23 Dec. 1980	
A	US,A, 4,485,045, Regen 27 Nov 1984	